From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 20 June 2000 (20.06.00)	
International application No. PCT/US99/25593	**Applicant's or agent's file reference** 11909.1WOU2
International filing date (day/month/year) 29 October 1999 (29.10.99)	**Priority date** (day/month/year) 30 October 1998 (30.10.98)
Applicant HOLTZMAN, Jordan, L.	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

26 May 2000 (26.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Kiwa Mpay

Telephone No.: (41-22) 338.83.38

KCW

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

DUE ATY Resp SR Aug 19, 2000
ATY IDS-US Sept- 19, 2000

To:
MERCHANT & GOULD P.C.
3100 Norwest Center
90 South Seventh Street
Minneapolis, Minnesota 55402-4131
UNITED STATES OF AMERICA

Applicant's or agent's file reference 11909.1WOU2 ✓	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 99/25593 ✓	International filing date (day/month/year) 29/10/1999
Applicant HOLTZMAN, Jordan, L.	

Date of mailing (day/month/year) 19/06/2000

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Véronique Baillou

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
11909.1WOU2		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/25593	29/10/1999	30/10/1998
Applicant		
HOLTZMAN, Jordan, L.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIO SEARCH REPORT

International application No.
PCT/US 99/25593

Box I Observati ns wh re certain claims w r f und unsearchabl (Continuation f item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 33 - 35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERN ONAL SEARCH REPORT

Inter. ...nal Application No
US 99/25593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US November 1998 (1998-11) HOLTZMAN J L ET AL: "A chaperon binds to b-amyloids: Implications for the etiology of Alzheimer's disease." Database accession no. PREV199900015705 XP002139087 abstract & 38TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CELL BIOLOGY;SAN FRANCISCO, CALIFORNIA, USA; DECEMBER 12-16, 1998, vol. 9, no. SUPPL., November 1998 (1998-11), page 106A Molecular Biology of the Cell Nov., 1998 ISSN: 1059-1524 --- -/--	1-3,12, 22,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	12. 06. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Goetz, M

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 780 587 A (POTTER HUNTINGTON) 14 July 1998 (1998-07-14) column 3, line 44 -column 10, line 6; claims 1-5 column 17, line 1-42 column 18, line 47 -column 19, line 44 column 21, line 34-67 column 22, line 31-34 claims 7,8; examples 13,14	4-7,36, 37
X	EP 0 783 104 A (ORIENTAL YEAST CO LTD) 9 July 1997 (1997-07-09) claim 8; example 2	36,37
X	IDA ET AL.: "Analysis of heterogeneous ßA4 Peptides in Human Cerebrospinal Fluid and Blood by a Newly Developed Sensitive Western Blot Assay" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996 (1996-09-13), pages 22908-22914, XP002138701 page 22909, right-hand column, last paragraph -page 22910, left-hand column, paragraph 2	36,37
A	WO 96 12736 A (POTTER HUNTINGTON ;HARVARD COLLEGE (US)) 2 May 1996 (1996-05-02) page 3, line 31 -page 4, line 12 page 10, line 11-16	1-37
A	MATSUBARA ET AL.: "Apolipoprotein J and Alzheimer's amyloid ß solubility" BIOCHEMICAL JOURNAL, vol. 316, 1996, pages 671-679, XP002138702 page 671, right-hand column, paragraph 1 page 678, right-hand column, last paragraph	1-37

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/25593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780587 A	14-07-1998	US 5506097 A	09-04-1996
		US 5338663 A	16-08-1994
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997
		WO 9612736 A	02-05-1996
		EP 0623146 A	09-11-1994
		JP 7509447 T	19-10-1995
		WO 9315112 A	05-08-1993
		EP 0546101 A	16-06-1993
		JP 6500560 T	20-01-1994
		WO 9203474 A	05-03-1992
EP 0783104 A	09-07-1997	JP 9178743 A	11-07-1997
WO 9612736 A	02-05-1996	US 5780587 A	14-07-1998
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
11909.1WOU2		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/25593	29/10/1999	30/10/1998
International Patent Classification (IPC) or national classification and IPC C07K14/47		
Applicant HOLTZMAN, Jordan, L.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/05/2000	16.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Goetz, M Telephone No. +49 89 2399 8697

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/25593

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-24 as originally filed

Claims, No.:

1-37 as originally filed

Drawings, sheets:

1/16-16/16 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 33 - 35.

because:

☒ the said international application, or the said claims Nos. 33 - 35 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 1 - 3, 8 - 35

	No:	Claims	4 - 7, 36, 37
Inventive step (IS)	Yes:	Claims	1 - 3, 8 - 35
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1 - 32, 36, 37
	No:	Claims	see section III.

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 33 - 35 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT), see section V below.

However, an opinion with respect to novelty / inventive step will be expressed on the basis of the alleged technical effects recited in the said claims.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive st p or industrial applicability; citations and explanations supporting such statem nt

1. Reference is made to the following documents:

   D1: Holtzman et al., 38th Annual Meeting of the American Society for Cell Biology, San Francisco, CA, USA, Mol.Biol.Cell Vol. 9/Suppl., November 1998, p. 106A
   D2: Matsubara et al., Biochem.J. 316, 1996, pp. 671-679
   D3: WO96/12736
   D4: US-A-5 750 349
   D5: EP-A-0 783 104
   D6: Ida et al., J.Biol.Chem. 271, 1996, pp. 22908-22914

2. **D1**, disclosing the complexation of chaperone Q2 with ß-amyloid and its correlation with a patient's susceptibility to Alzheimer's disease (see the wording of the abstract), has been published after the validly claimed priority date of 30/10/98 and does not therefore constitute relevant prior art.

   D2 and **D3** describe the role of apo-E, apo-AI, apo-AII, apoE2-E4, α-antichymotrypsin, transthyretin and apo-J as molecular chaperones for ß-

amyloid (**D2**, page 671 / right column / lines 1 - 3, page 678 / right column / last paragraph; **D3**, page 3 / line 31 - page 4 / line 12, page 10 / lines 11 - 16)

However, none of the currently available documents neither discloses nor suggests the existence of a complex comprised of the Q2 chaperone and ß-amyloid protein. Hence, the subject-matter of claims 1 - 3 and 8 - 21 is novel and involves an inventive step and therefore complies with Art. 33(2) and (3) PCT.

As the detection of a Q2 / ß-amyloid complex apparently enabled the inventors to establish a correlation between Q2 levels and Alzheimer's disease, the subject-matter of claims 22 - 35, based on said correlation, is also novel and involves an inventive step and therefore complies with Art. 33(2) and (3) PCT.

3. Page 7 / lines 23 - 24 of the present description state that polyclonal or monoclonal antibodies recognizing either Q2 or ß-amyloid are known in the art; indeed, documents **D4**, **D5** and **D6** disclose the preparation of various monoclonal antibodies against several isoforms of ß-amyloid protein (**D4**, column 3 / line 44 - column 10 / line 6, claims 1 - 5; **D5**, example 2, claim 8; **D6**, page 22909 / right column / section *"Results"*).

According to page 7 / lines 21 - 23 of the present description, the Q2 / Chaperone complex can be recognized by such known antibodies; hence, the subject-matter of claims 36 and 37 is anticipated by the antibodies according to **D4**, **D5** and **D6**.

Claims 36 and 37 do not therefore comply with the requirements according to Art. 33(2) PCT.

3.1. Moreover, **D4** discloses various immunoassays for the detection of ß-amyloid protein in CSF of Alzheimer patients (**D4**, column 17 / lines 1 - 42, column 18 /line 47 - column 19 / line 44, column 21 / lines 34 - 67, column 22 / lines 31 - 34, examples 13 and 14, claims 7 - 8).

Since, according to claim 4 and page 7 / lines 21 - 31 of the present description, the Q2 / chaperone complex can be detected by performing assays which recognize ß-amyloid protein, the immunoassays according to **D4** anticipate the

subject-matter of claims 4 - 7 which do not therefore comply with the requirements according to Art. 33(2) PCT.

4. As a precautionary measure, the Applicant is notified that for the assessment of the presently worded claims 33 - 35 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also depend on the formulation of the claim. The EPO, for example, does not recognise as industrially applicable claims to a method of treating the human or animal body by diagnosis, therapy or surgery, the use of a compound in such a treatment, but will allow claims to a known compound for first use in said treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

Claim 1 does not comply with Art. 6 PCT, as the meaning of the term *"Q2"* alone is unclear; the objection would not have been raised if claim 1 would recite a *"chaperone Q2"*.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US November 1998 (1998-11) HOLTZMAN J L ET AL: "A chaperon binds to b-amyloids: Implications for the etiology of Alzheimer's disease." Database accession no. PREV199900015705 XP002139087 abstract & 38TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CELL BIOLOGY;SAN FRANCISCO, CALIFORNIA, USA; DECEMBER 12-16, 1998, vol. 9, no. SUPPL., November 1998 (1998-11), page 106A Molecular Biology of the Cell Nov., 1998 ISSN: 1059-1524 --- -/--	1-3,12, 22,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	19. 06. 00
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Goetz, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25593

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 780 587 A (POTTER HUNTINGTON) 14 July 1998 (1998-07-14) column 3, line 44 -column 10, line 6; claims 1-5 column 17, line 1-42 column 18, line 47 -column 19, line 44 column 21, line 34-67 column 22, line 31-34 claims 7,8; examples 13,14	4-7,36, 37
X	EP 0 783 104 A (ORIENTAL YEAST CO LTD) 9 July 1997 (1997-07-09) claim 8; example 2	36,37
X	IDA ET AL.: "Analysis of heterogeneous βA4 Peptides in Human Cerebrospinal Fluid and Blood by a Newly Developed Sensitive Western Blot Assay" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996 (1996-09-13), pages 22908-22914, XP002138701 page 22909, right-hand column, last paragraph -page 22910, left-hand column, paragraph 2	36,37
A	WO 96 12736 A (POTTER HUNTINGTON ;HARVARD COLLEGE (US)) 2 May 1996 (1996-05-02) page 3, line 31 -page 4, line 12 page 10, line 11-16	1-37
A	MATSUBARA ET AL.: "Apolipoprotein J and Alzheimer's amyloid β solubility" BIOCHEMICAL JOURNAL, vol. 316, 1996, pages 671-679, XP002138702 page 671, right-hand column, paragraph 1 page 678, right-hand column, last paragraph	1-37

INTERNATIONAL SEARCH REPORT

[redacted]tional application No.
PCT/US 99/25593

Box I Ob rvations where certain laims w re found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 33 - 35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/25593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780587 A	14-07-1998	US 5506097 A	09-04-1996
		US 5338663 A	16-08-1994
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997
		WO 9612736 A	02-05-1996
		EP 0623146 A	09-11-1994
		JP 7509447 T	19-10-1995
		WO 9315112 A	05-08-1993
		EP 0546101 A	16-06-1993
		JP 6500560 T	20-01-1994
		WO 9203474 A	05-03-1992
EP 0783104 A	09-07-1997	JP 9178743 A	11-07-1997
WO 9612736 A	02-05-1996	US 5780587 A	14-07-1998
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

FEB 16 2000

DAIGNAULT, Ronald, A.
Merchant & Gould P.C.
3100 Norwest Center
90 South Seventh Street
Minneapolis, MN 55402-4131
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 31 January 2000 (31.01.00)	
Applicant's or agent's file reference 11909.1WOU2	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/25593	International filing date (day/month/year) 29 October 1999 (29.10.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 30 October 1998 (30.10.98)
Applicant HOLTZMAN, Jordan, L.	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
30 Octo 1998 (30.10.98)	60/106,398	US	24 Janu 2000 (24.01.00)
10 Marc 1999 (10.03.99)	60/123,564	US	27 Janu 2000 (27.01.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Taïeb Akremi

Telephone No. (41-22) 338.83.38

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
G01N 33/68, C07K 14/47

A3

(11) International Publication Number: WO 00/26251

(43) International Publication Date: 11 May 2000 (11.05.00)

(21) International Application Number: PCT/US99/25593

(22) International Filing Date: 29 October 1999 (29.10.99)

(30) Priority Data:

60/106,398	30 October 1998 (30.10.98)	US
60/123,564	10 March 1999 (10.03.99)	US

(71)(72) Applicant and Inventor: HOLTZMAN, Jordan, L. [US/US]; 4710 Girard Avenue South, Minneapolis, MN 55409 (US).

(74) Agent: DAIGNAULT, Ronald, A.; Merchant & Gould P.C., 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402–4131 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
10 August 2000 (10.08.00)

(54) Title: A COMPLEX OF A CHAPERONE WITH ß–AMYLOID AND METHODS EMPLOYING THIS COMPLEX

(57) Abstract

A chaperone protein Q2 and ß–amyloid can form a complex. This complex can be detected in a biological sample, such as, for example, tissues or fluids from a mammal. Q2 levels can also be detected in a biological sample. A method for determining the Q2 level in a biological sample and comparing that level to a normal Q2 level can be used to detect, screen, diagnose, or otherwise determine a person's susceptibility to Alzheimer's disease such as, for example, the presence or absence of Alzheimer's disease, of symptoms of this disease, of factors leading to or associated with this disease, of likelihood of developing this disease, and the like. In one embodiment, a decline in Q2 level correlates to an increased likelihood of developing Alzheimer's disease. In another embodiment, a decline in Q2 level correlates to an increase in ß–amyloid aggregation. The method may further include screening for an apolipoprotein E genetype, which is associated with Alzheimer's disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US November 1998 (1998-11) HOLTZMAN J L ET AL: "A chaperon binds to b-amyloids: Implications for the etiology of Alzheimer's disease." Database accession no. PREV199900015705 XP002139087 abstract & 38TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CELL BIOLOGY;SAN FRANCISCO, CALIFORNIA, USA; DECEMBER 12-16, 1998, vol. 9, no. SUPPL., November 1998 (1998-11), page 106A Molecular Biology of the Cell Nov., 1998 ISSN: 1059-1524 --- -/—	1-3,12, 22,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	19. 06. 00
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Goetz, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25593

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 780 587 A (POTTER HUNTINGTON) 14 July 1998 (1998-07-14) column 3, line 44 -column 10, line 6; claims 1-5 column 17, line 1-42 column 18, line 47 -column 19, line 44 column 21, line 34-67 column 22, line 31-34 claims 7,8; examples 13,14	4-7,36, 37
X	EP 0 783 104 A (ORIENTAL YEAST CO LTD) 9 July 1997 (1997-07-09) claim 8; example 2	36,37
X	IDA ET AL.: "Analysis of heterogeneous βA4 Peptides in Human Cerebrospinal Fluid and Blood by a Newly Developed Sensitive Western Blot Assay" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996 (1996-09-13), pages 22908-22914, XP002138701 page 22909, right-hand column, last paragraph -page 22910, left-hand column, paragraph 2	36,37
A	WO 96 12736 A (POTTER HUNTINGTON ;HARVARD COLLEGE (US)) 2 May 1996 (1996-05-02) page 3, line 31 -page 4, line 12 page 10, line 11-16	1-37
A	MATSUBARA ET AL.: "Apolipoprotein J and Alzheimer's amyloid β solubility" BIOCHEMICAL JOURNAL, vol. 316, 1996, pages 671-679, XP002138702 page 671, right-hand column, paragraph 1 page 678, right-hand column, last paragraph	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/25593

Box I Obs rvations where rtain claims were found un earchable (Continuation of item 1 of first sh t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 33 - 35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/25593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780587 A	14-07-1998	US 5506097 A	09-04-1996
		US 5338663 A	16-08-1994
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997
		WO 9612736 A	02-05-1996
		EP 0623146 A	09-11-1994
		JP 7509447 T	19-10-1995
		WO 9315112 A	05-08-1993
		EP 0546101 A	16-06-1993
		JP 6500560 T	20-01-1994
		WO 9203474 A	05-03-1992
EP 0783104 A	09-07-1997	JP 9178743 A	11-07-1997
WO 9612736 A	02-05-1996	US 5780587 A	14-07-1998
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 14/47, G01N 33/53

A2

(11) International Publication Number: WO 00/26251

(43) International Publication Date: 11 May 2000 (11.05.00)

(21) International Application Number: PCT/US99/25593

(22) International Filing Date: 29 October 1999 (29.10.99)

(30) Priority Data:

60/106,398	30 October 1998 (30.10.98)	US
60/123,564	10 March 1999 (10.03.99)	US

(71)(72) Applicant and Inventor: HOLTZMAN, Jordan, L. [US/US]; 4710 Girard Avenue South, Minneapolis, MN 55409 (US).

(74) Agent: DAIGNAULT, Ronald, A.; Merchant & Gould P.C., 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402–4131 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: A COMPLEX OF A CHAPERONE WITH ß–AMYLOID AND METHODS EMPLOYING THIS COMPLEX

(57) Abstract

A chaperone protein Q2 and ß–amyloid can form a complex. This complex can be detected in a biological sample, such as, for example, tissues or fluids from a mammal. Q2 levels can also be detected in a biological sample. A method for determining the Q2 level in a biological sample and comparing that level to a normal Q2 level can be used to detect, screen, diagnose, or otherwise determine a person's susceptibility to Alzheimer's disease such as, for example, the presence or absence of Alzheimer's disease, of symptoms of this disease, of factors leading to or associated with this disease, of likelihood of developing this disease, and the like. In one embodiment, a decline in Q2 level correlates to an increased likelihood of developing Alzheimer's disease. In another embodiment, a decline in Q2 level correlates to an increase in ß–amyloid aggregation. The method may further include screening for an apolipoprotein E genetype, which is associated with Alzheimer's disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

A COMPLEX OF A CHAPERONE WITH β–AMYLOID AND METHODS EMPLOYING THIS COMPLEX

Background of the Invention

Numerous physiological problems, such as a loss in muscle mass, a failure of the immune system, decreases in the maximal synthesis and release of hormones (e.g. insulin or growth hormone), loss of renal function, and decreases in cognitive skills occur with aging. These problems lead to an overall decline in functional capacity. Several models have been advanced to explain these age–related physiological problems. Such models include, for example, increased programmed cell death, i.e. apoptosis; accumulation of oxidant damage; failure of the cell to maintain the telomeres at the ends of the chromosomes; and defects in responding to stress. The observed age–related defects in responding to stress may involve chaperone proteins.

At the cellular level the most important stress proteins are the chaperones. Chaperones play an important role in cellular function. They help to realign proteins into their native state, thereby renaturing damaged proteins and aid the final steps of protein folding by directing newly synthesized proteins into their final, optimal structure. Chaperones also help stabilize the final protein product, such as by the formation of intra– and intermolecular disulfide bonds. One such family of chaperones is known as thiol:protein disulfide oxidoreductases (TPDOs). Studies of the stress proteins and chaperones support the concept that many of the age–related functional declines are associated with decreases in the activity of the chaperone systems. Decreased levels and activity of chaperones can result in increased formation of improperly folded and insoluble masses of proteins.

Insoluble masses, or plaques, of the β–amyloid protein, a 38 to 43 amino acid peptide derived from the amyloid precursor protein, form in the brain of older persons suffering from Alzheimer's disease. Amyloid precursor protein is an intrinsic membrane protein that is synthesized in the endoplasmic reticulum. During synthesis and insertion into the plasma membrane, β–amyloid is cleaved off the amyloid precursor protein and secreted into the intercellular space.

In physiological solutions β–amyloid readily aggregates to form plaques characteristic of Alzheimer's disease. However, Alzheimer's disease is complex and involves more than mere overexpression of the β–amyloid peptide. The neuropathology of Alzheimer's disease is characterized by extensive neuronal cell

loss and deposition of numerous senile plaques and neurofibrillary tangles in the cerebral cortex. Although small numbers of classic senile plaques develop in the normal brain with age, large numbers of the plaques are found almost exclusively in Alzheimer's patients.

One study showed that when cerebrospinal fluid is added to β–amyloid, β–amyloid does not aggregate, suggesting that cerebrospinal fluid includes a component that inhibits β–amyloid aggregation. This indicates that cerebrospinal fluid of subjects that are free of Alzheimer's disease may include a component that prevents formation of senile plaques. This component could be a chaperone. Thus, it is desirable to better characterize the role of chaperones in processing of amyloid precursor protein, forming β–amyloid plaques, and Alzheimer's disease. Proper folding or processing of the amyloid precursor protein or β–amyloid may be involved in the etiology of Alzheimer's disease.

Alternatively, a patient with Alzheimer's disease may have a protein that enhances nucleation of β–amyloid plaques. One theory suggests that apolipoprotein E may play a role in Alzheimer's disease. Apolipoprotein E exists in at least 3 allelic forms known as $apoE_2$, $apoE_3$, and $apoE_4$. Evidence indicates that a person who has at least one allele of apolipoprotein E_4 ($apoE_4$) is more susceptible to Alzheimer's disease, suggesting that the protein product of $apoE_4$ may play a role in Alzheimer's disease. Moir et al., Biochemistry, **38**: 4595–4603 (1999). For example, $apoE_4$ may contribute to the nucleation or formation of β–amyloid plaques by contributing to the aggregation of β–amyloid.

Previously, Alzheimer's disease studies have focused on overproduction of β–amyloid. For instance, many laboratories have investigated the role of proteases involved in cleaving the precursor protein to produce β–amyloid. Yet a number of studies have shown that, with the exception of some rare genetic forms of early onset Alzheimer's disease and the early Alzheimer's disease seen with Down's syndrome, patients with Alzheimer's disease actually have lower concentrations of β–amyloid in their cerebrospinal fluid than age–matched controls. Further, a recent study of transgenic mice having an amyloid precursor protein gene lacking the Kunitz–protease inhibitor domain showed that the increased concentration of β–amyloid cannot be explained by a rise in expression of amyloid precursor protein, which appeared to remain unchanged with age. These studies indicate that β–amyloid levels alone are not enough to explain Alzheimer's disease. Thus, it is desirable to

better characterize the role of chaperones and processing of amyloid precursor protein in forming β–amyloid plaques and in Alzheimer's disease. Proper folding or processing of the amyloid precursor protein or β–amyloid may be involved in the etiology of Alzheimer's disease.

Furthermore, at present the only method to detect a propensity for formation of β–amyloid plaque or Alzheimer's disease or the presence of such plaques or disease includes dissection of the brain or culturing of brain cells of the subject. Such invasive procedures are, of course, undesirable for most subjects. This is particularly so, since as outlined above, even after such dissection, it previously would have been unclear how to test for certain factors leading to plaque formation or disease. This demonstrates a need for a method to detect the propensity for or presence of plaques or disease in a living, intact subject.

Summary of the Invention

The present invention generally relates to a composition and method of detecting this composition that meet the needs described above. The present invention includes an isolated complex of a chaperone and β–amyloid protein. Preferably the complex includes the chaperone Q2 and β–amyloid. The complex can be natural or produced by biotechnological methods and can be purified.

The present invention also includes a method for detecting Q2 levels. The method can include an immunoassay, a method of chemical detection, a method of physical detection, or the like. Preferably the method employs an immunoassay. The method for detecting the Q2 levels can be employed as a reagent in a clinical or scientific laboratory, as a method for determining the propensity of a biological system to form plaques of β–amyloid or for determining the presence or likelihood of Alzheimer's disease or symptoms associated with Alzheimer's disease.

Brief Description of the Drawings

Figure 1 illustrates rat weights as a function of age.

Figure 2 illustrates rat liver weights as a function of age.

Figure 3 illustrates the amount of microsomal protein per gram of liver as a function of age.

Figure 4 illustrates a survival rate of rats as a function of age.

Figure 5 illustrates a mortality rate of rats as a function of age.

Figure 6 illustrates rat hepatic, microsomal Q5 levels as a function of age.

Figure 7 illustrates rat stress–responsive levels of Q5 as a function of age.

Figure 8 illustrates rat hepatic, microsomal Q2 levels as a function of age.

Figure 9 illustrates rat stress–responsive levels of Q2 as a function of age.

Figure 10 illustrates rat hepatic, microsomal Erp72 levels as a function of age.

Figure 11 illustrates rat hepatic, microsomal BiP levels as a function of age.

Figure 12 illustrates rat hepatic, microsomal calnexin levels as a function of age.

Figure 13 illustrates rat hepatic, microsomal calreticulin levels as a function of age.

Figure 14A illustrates an immunoblot of biological samples with polyclonal antibodies to Q2.

Figure 14B illustrates an immunoblot of biological samples with monoclonal antibodies to β–amyloid 1–42.

Figure 15A illustrates another immunoblot of biological samples with polyclonal antibodies to Q2.

Figure 15B illustrates another immunoblot of biological samples with polyclonal antibodies to β–amyloid 1–42.

Figure 16 illustrates cerebrospinal fluid concentrations of Q2 compared to senile plaque scores measured from participants in the nun study described in Example 5.

Detailed Description of the Invention

The present invention relates to a complex of a chaperone protein with β–amyloid, methods for detecting levels of Q2, methods for employing such detection in diagnosis of Alzheimer's disease, and methods of enhancing levels of this chaperone protein to treat or prevent Alzheimer's disease. In particular, the present invention is directed to an isolated complex of β–amyloid and the chaperone Q2, methods to detect levels of Q2 in, for example, clinical or autopsy samples, methods to diagnose Alzheimer's disease based on detecting a decrease in concentration of Q2 in clinical samples, such as cerebrospinal fluid, and methods of treating Alzheimer's disease by stimulating Q2 production or by administering Q2.

Alzheimer's Disease and β–Amyloid

Alzheimer's disease includes formation of β–amyloid plaques in the brains of subjects with the disease. The plaques form from aggregates of β–amyloid, which form from cleavage of the amyloid precursor protein and secretion into the intercellular space. β–Amyloid freely aggregates in solution in laboratory systems and forms aggregates similar to the plaques formed in Alzheimer's disease.

Amyloid precursor protein, β–amyloid, and various fragments of β–amyloid have been characterized. Known features of amyloid precursor protein and β–amyloid include mammalian genes encoding them, recombinant expression systems (e.g. vectors, plasmids, and the like) for these proteins, methods of producing these proteins, protein sequences and structures, and proteases that cleave the amyloid precursor to β–amyloid. Neither amyloid precursor protein, β–amyloid, nor any of the various fragments of β–amyloid have previously been observed to interact with a chaperone protein.

β–Amyloid can be made and/or isolated in a variety of forms. The most prevalent form of β–amyloid in mammalian tissues is β–amyloid 1–42, where the numbering represents the number of amino acids starting at the amino terminus of complete β–amyloid, which has from 38–43 amino acids depending on species. The second most prevalent form of β–amyloid in mammalian tissues is β–amyloid 1–38.

β–Amyloids have been produced by chemical and/or biotechnical methods, characterized, and shown to have many of the properties of complete β–amyloid. β–Amyloid as used herein refers to all of the various forms of β–amyloid, including glycosylated, nonglycosylated, forms of various lengths, and the like.

Chaperones

Chaperones (also known as chaperone proteins, and including chaperonins and certain heat shock proteins) catalyze folding, formation of tertiary structure, formation of quaternary structure, and/or other processing to make an active protein. As described herein, several chaperones can decline in level with age and can be correlated with age–related diseases and disorders. In particular, tissue levels of one or more of the chaperones BiP, calreticulin, calnexin, Erp72, Q2, and Q5 can decrease with age of a mammal, such as a rodent or a human, and can correlate with a disease. Chaperones include a family known as a thiol:protein disulfide

oxidoreductase (TPDO). A TPDO represents a preferred chaperone of the present invention. A preferred TPDO is TPDO–Q2. TPDO–Q2 has also been called ERp57 and GRp58. As used herein, Q2 refers to any of the common names for this protein, including TPDO–Q2, ERp57, and GRp58 and all naturally occurring variant forms of this protein, including glycosylated and nonglycosylated forms.

Chaperones are, in general, well studied and/or characterized proteins. Well characterized features of numerous chaperones include the genes encoding them in organisms ranging from bacteria to humans, recombinant expression systems (e.g. vectors, plasmids, and the like) for these proteins, methods of producing these proteins, protein sequences and structures, certain protein substrates, and certain biological functions. Chaperones have not previously been observed to decrease with age of a mammal nor have they been implicated in Alzheimer's disease or formation of β–amyloid plaques.

A decline in chaperone synthesis can result from a decline in chaperone transcription. To determine whether the amount of chaperones declines with age in tissues and/or fluids relevant to Alzheimer's disease and/or β–amyloid plaque formation, the concentration of specific mRNAs for various chaperones can be studied by known methods, for example, standard hybridization techniques.

A Complex of Chaperone Q2 and β–Amyloid

Although other chaperones may be present in cerebrospinal fluid, only Q2 has been identified in cerebrospinal fluid. Q2 levels can decrease relative to normal Q2 levels in mammalian tissue with age. "The term normal Q2 levels" as used herein includes the mean concentration of Q2 that can typically be found in cerebrospinal fluid from a control population. Suitable control populations include, for example, young people, elderly people without Alzheimer's disease, and the like. A normal Q2 level can be about 27 ± 2 ng/ml.

Q2 can form a complex with β–amyloid. This complex can be isolated from cerebrospinal fluid and created in the laboratory. Q2 and β–amyloid are believed to form a strongly bound complex. For instance, immunoblots evidence very little dissociation of the complex. The complex of Q2 and β–amyloid is believed to be an intermediate in normal processing of amyloid precursor in subjects not suffering from Alzheimer's disease. A decrease in the level of Q2 relative to normal Q2 levels in an aging animal can lead to decreased amounts of the complex and increased amounts of free β–amyloid. Increased amounts of β–amyloid can give

rise to increased depositions of amyloid plaques associated with Alzheimer's disease.

The complex of Q2 and β–amyloid can be isolated from cerebrospinal fluid, purified, and characterized by common methods of protein chemistry. For example, the complex can be isolated and/or purified by affinity chromatography with a system having affinity for one or either of Q2 or β–amyloid. The complex can then be further purified by other forms of chromatography, such as separation on a Sephacryl column and/or a monoQ column. Following isolation and/or purification, the complex can be characterized by employing standard methods such as peptide mapping, sequencing, the PAS reaction (periodic acid–Schiff reaction), and matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF).

The complex of Q2 and β–amyloid can be glycosylated in natural systems. Under most circumstances outside the complex, neither Q2 nor β–amyloid appear to be glycosylated. Typically the complex is glycosylated, for example, N–glycosylated on asparagine residues with complex oligosaccharides.

Detecting Q2 Levels

The complex of Q2 and β–amyloid and the unbound components Q2 and β–amyloid can be detected by methods known for detecting proteins, sugars, and/or glycosylated proteins. The complex has a molecular weight of about 62 kD on gently or moderately denaturing gel electrophoresis and is recognized by antibodies to either Q2 or β–amyloid. Polyclonal or monoclonal antibodies recognizing either Q2 or β–amyloid are known in the art and can be produced by standard methods. Such antibodies can be labeled or otherwise employed in standard immunoassays. Preferred standard immunoassays include ELISA assays, immunoblots, sandwich assays, enhanced chemiluminescence, and the like. Additional methods for detecting the complex or its unbound components include fluorescence polarization assay, standard protein purification by column and affinity chromatography, proteolytic cleavage followed by Edman degradation analysis, MALDI–TOF, and time–of–flight mass spectrometry.

Methods for detecting Q2 levels in biological samples can be employed in a method for detecting, determining, examining, diagnosing, screening for, or otherwise assessing a patient's or subject's susceptibility to Alzheimer's disease. The term "susceptibility to Alzheimer's disease" as used herein includes the presence

or absence of Alzheimer's disease, of symptoms of this disease, of factors leading to or associated with this disease, of likelihood of developing this disease in a subject or patient, and the like. As used herein, factors leading to or associated with this disease include formation of β–amyloid plagues, disorders in processing of amyloid precursor protein, and the like.

As used herein biological samples include biological material such as a tissue, cell, or fluid sample from an animal or human in need of being screened for or suspected of being susceptible to or suffering from deposition of β–amyloid plaques, disorders in processing of amyloid precursor protein, Alzheimer's disease, likelihood of developing Alzheimer's disease, and the like. Biological samples can also include laboratory samples from an experimental animal, cell, or culture being examined for their propensity to form β–amyloid plaques or aggregates or to improperly process amyloid precursor protein. The biological sample including, for example, tissues or cells, such as liver, platelets, serum, or skin, can be recovered from a living animal or patient by methods such as needle biopsy, venapuncture, or skin scraping. In one aspect of the invention, the biological sample includes biological material from the central nervous system of a human or an animal. A preferred biological sample includes biological material from cerebrospinal fluid for assessing the susceptibility to Alzheimer's disease in living animals or patients. Cerebrospinal fluid can be recovered by spinal tap, during surgical procedures, or by any of the variety of methods known to those of skill in the art.

Generally the decline in Q2 levels relative to normal Q2 correlates to the susceptibility to Alzheimer's disease. The level of Q2 or Q2 level includes the level of free Q2 in the biological sample, the level of Q2 bound in complex with β–amyloid, the levels of free Q2 plus bound Q2, and preferably total Q2. A decline in the Q2 level correlates to an increased susceptibility to Alzheimer's disease. An increased susceptibility, as used herein, refers to it being more probable than not that Alzheimer's disease, at least one symptom of the disease, at least one factor leading to or associated with this disease, likelihood of developing this disease, or the like is present in a patient or subject. For example, in one embodiment a decline in Q2 level of at least 35% relative to a normal Q2 level correlates with a 100% likelihood of developing Alzheimer's disease. In another embodiment, the aggregation of β–amyloid in an animal or human can be detected by determining the level of Q2 in the biological sample. A decline in Q2 levels relative to normal Q2 levels correlates to and indicates an increased susceptibility to an increase in β–amyloid aggregation.

By detecting the aggregation of β–amyloid, the formation of β–amyloid plaques is also detected.

The susceptibility to Alzheimer's disease may also be determined by correlating the level of free Q2, the level of β–amyloid, and the level of Q2: β–amyloid in complex. A relevant decline in Q2 levels relative to normal Q2 levels includes a decline of about 35%, preferably about 50%, more preferably about 65%. Such a relevant decline correlates to an increased susceptibility to Alzheimer's disease, for example, to factors leading to or associated with Alzheimer's disease, particularly β–amyloid plaque formation; to Alzheimer's disease; to symptoms associated with Alzheimer's disease; to developing Alzheimer's disease; or the like.

The decline in Q2 levels relative to normal Q2 levels can be correlated with symptoms characteristic of Alzheimer's disease by comparing Q2 levels with neuropsychiatric function measurements. Neuropsychiatric function measurements can be made by conducting, for example, a neuropsychiatric evaluation, a complete history, and a physical examination and evaluating this information based on criteria defined by, for example, DSMIII–R and NinCDS–ADRDA.

In some instances, individuals who have Q2 levels of about normal can also be susceptible to Alzheimer's disease. For example, individuals having normal Q2 levels but an $apoE_4$ allele have an increased susceptibility to Alzheimer's disease. There may be other risk factors for Alzheimer's disease that result in an increased susceptibility to Alzheimer's disease in the presence of normal Q2 levels.

Enhancing Q2 Levels

Q2 levels can be enhanced by administering a drug suitable for increasing Q2 expression or by administering Q2. Administering Q2 includes administering Q2 in a form or precursor of this protein, in a manner that increases Q2 levels in, for example, the cerebrospinal fluid. Enhancing Q2 levels can increase the amount of complex of Q2 with β–amyloid, increase the level of appropriately processed β–amyloid and/or amyloid precursor protein, and/or decrease the amount of β–amyloid plaque in a subject, and the like.

Q2 can be administered in a pharmaceutically acceptable vehicle to the cerebrospinal fluid by methods such as injection, by an intracerebroventricular pump, and the like. Alternatively, Q2 can be administered as a precursor gene or vector encoding Q2, which can be targeted for the central nervous system and

provide expression and increased levels of Q2. Vectors that can encode Q2 and that will express protein in and/or target brain cells are known to those of skill in the art.

Preferably Q2 or a drug suitable for enhancing Q2 expression is administered in an amount effective to increase Q2 level in, for example, cerebrospinal fluid, to increase the amount of complex of Q2 with β–amyloid, to increase the level of appropriately processed β–amyloid and/or amyloid precursor protein, and/or to decrease the amount of β–amyloid plaque in a subject, or the like. Further, such administration can affect the course or outcome of Alzheimer's disease. An "effective amount" of Q2 or a drug suitable for enhancing Q2 expression is an amount sufficient to prevent, treat, reduce, and/or ameliorate the symptoms and/or underlying causes of Alzheimer's disease including the level of Q2, the level of appropriately processed β–amyloid and/or amyloid precursor protein, and/or the amount of β–amyloid plaque in a subject, or the like. In some instances, an "effective amount" is sufficient to eliminate the symptoms of the disease and, perhaps, overcome the disease itself. In the context of the present invention, the terms "treat" and "therapy" and the like refer to alleviate, slow the progression, prophylaxis, attenuation, or cure of existing disease. "Prevent" as used herein, refers to putting off, delaying, slowing, inhibiting, or otherwise stopping, reducing, or ameliorating the onset of such brain diseases or disorders.

The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

EXAMPLES

Example 1 – – Characterization of the Effect of Aging on Chaperone Concentration in Animal Hepatic Microsomes

The effect of aging on chaperone concentration was determined to demonstrate a decrease in concentration that can be linked to age–related disorders such as plaque deposition or Alzheimer's disease.

Materials and Methods

The animals used in this study were male, specific pathogen free, Sprague–Dawley rats purchased at age 21 days from Harlan Laboratories (Madison, WI). For

the remainder of their lives, they were housed in a windowless, controlled environment, "state–of–the–art," barrier facility with a constant temperature of 22° C ± 2° and a 12 hour on and 12 hour off light cycle. The humidity was maintained at 50% ± 20%. Two animals were housed in each cage and the animals had free access to food and water. Each cage containing test animals was equipped with an air filter cover. Ten percent of the cages did not have filters. The animals in the cages without filters were maintained as "sentinels" to determine whether there was any break in the sterile conditions. These animals were routinely replaced with new batches of young animals. They did not show an increase in mortality, indicating the colony was pathogen free.

All animals were removed from their cage once a week when the cages were cleaned and every four to six weeks when the animals were weighed. These procedures were performed under clean conditions. Any person who entered the animal room wore sterile gloves, gowns, and masks. The animals were fed a diet based on the AIN76A recommendations with the exception that the carbohydrate was supplied as 40% starch and 25% sucrose (BioServe, Frenchtown, NJ). The carbohydrate composition was changed to accommodate a preference for the diet to consist of pellets instead of a powder and to reduce the incidence of the obesity which is associated with high sucrose diets. Potassium citrate (6.5%) was added to prevent vascular disease. The dietary conditions met NRC nutritional standards for the adult rat. (NRC 1978).

The animals reached and maintained an adult weight of about 600g (Figure 1). However, after the age of 750 days, their average weights declined to 475g. This decline in the average weight of the animals that survived to the end of the study was the same as the average weight of the total population. The average adult liver weight was 17.5g and remained there for their entire adult lives (Figure 2). The milligram of microsomal protein per gram of liver showed no significant change with age (Figure 3). There was no significant mortality until the age of about 500 days (Figure 4). After 500 days, the mortality rate showed a Gompertz effect with a doubling every 96.1 days (Figure 5). Humans show a similar doubling in mortality every decade.

For the first 18 months, six animals were killed at six week intervals. For the remaining 12 months, the animals were killed at three month intervals. Tissue samples were obtained from approximately one–third of the animals. The remainder died from natural causes.

Microsomes were prepared by differential centrifugation according to Srivastava et al., J. Biol. Chem., **265**:8392–99 (1990). Microsomal suspensions were frozen and maintained at −70° C for the determination of the concentration of chaperones. The following chaperones were examined: BiP, calreticulin, calnexin, ERp72, Q2, and Q5. Chaperones were prepared by methods known in the art. The microsomal content of chaperones was determined by immunoblotting according to Zhou et al., Arch. Biochem. Biophys., **322**:390–94 (1995); Zhou et al. (1996); and Chen et al., Biochemistry, **25**: 8299–8306 (1996). A pooled microsomal suspension was run on each gel and used as a standard. The chaperone content of the microsomes was calibrated against purified chaperones. The immunoassays were run in the linear range for the individual chaperones.

Chaperones were prepared by methods known in the art. Srivastava et al., J. Biol. Chem., **265**: 8392–99 (1991); Chen et al., Biochemistry, **25**: 8299–8306 (1996).

Immunoblotting was done according to the method of Towbin et al., Proc. Natl. Acad. Sci. USA, **76**: 4350–4354 (1979). See also Srivastava et al., J. Biol. Chem., **265**: 8392–8399 (1991); Zhou et al., Arch. Biochem. Biophys., **322**: 390–394 (1995); Zhou et al., Chem. Res. Toxicol., **9**: 1176–1182 (1996); and Chen et al., Biochemistry, **25**: 8299–8306 (1996). The proteins were first separated by SDS-PAGE. Laemmli, Nature, **227**: 680–685 (1970). The proteins then were transferred to PVDF membranes (Immobilon P, Millipore Corp), and the membranes were reacted with chicken anti–chaperon antibodies. This was followed by goat anti–chicken IgY antibody coupled to alkaline phosphatase. The indicator dye used is a combination of nitroblue tetrazolium and 5–bromo–4–chloro–3–indolyl phosphate (BioRad, Richmond CA). The density of the bands was determined on a flat bed scanner (UMAX, Taiwan, R.O.C.) and analyzed using NIH Image (version 1.61) on a Power PC (Apple Computer). The concentration was determined by comparison to various concentrations corresponding to the average density of three channels on each gel containing a suspension of reference microsomes. The microsomal standards were calibrated against several concentrations of the purified proteins. The determinations of the chaperones in the microsomes were all within the linear range of the immunoassays.

Polyclonal antibodies to all chaperones, except for BiP, were developed in laying hens as described by Damiani et al., J. Biol. Chem.,**263**:340–343 (1988) and Chen et al., Biochemistry, **25**: 8299–8306 (1996). The specificity of these

antibodies has been verified in previous studies. Srivastava et al., J. Biol. Chem., **265**: 8392–8399 (1991); Zhou et al., Arch. Biochem. Biophys., **322**: 390–394 (1995); Zhou et al., Chem. Res. Toxicol., **9**: 1176–1182 (1996); Chen et al., Biochemistry, **25**, 8299–8306 (1996). Antibodies to BiP were obtained from StressGen (Vancouver, BC, Canada). Additionally, the antibodies prepared to calreticulin also were used to probe for calnexin because the lumenal portion of calnexin is highly homologous to calreticulin. Zhou et al. (1996); Chen et al. (1996); Cala et al. (1993).

Results

These studies yielded the results reported in Table 1 and in Figures 1–13. In particular, Figure 6 shows a decline in Q5 concentration as a rat ages; Figure 7 shows a decline in Q5 concentration for stress responsiveness as a rat ages; Figure 8 shows a decline in Q2 concentration as a rat ages; Figure 9 shows a decline in Q2 concentration for stress responsiveness as a rat ages; Figure 10 shows a decline in ERp72 concentration as a rat ages.

Figure 11 shows a decline in BiP concentration as a rat ages; Figure 12 shows a decline in calnexin concentration as a rat ages; and Figure 13 shows no significant decline in calreticulin levels as a rat ages.

TABLE 1: Effect of Age and Stress on the Concentrations of the Various ER Chaperons.

Chaperon	Peak Concentration µg/mg Prot	Concentration @ 874 days µg/mg Prot	Constitutive Decline % (R^2)	Show Stress Response	Stress Decline %
BiP	80.0	48.5	39 (0.9586)	Yes	50
Calnexin	57.4	40.5	29 (0.9398)	No	—
Calreticulin	7.6	4.8	8	No	—
ERp72	141	100	30 (0.8840)	No	—
Q2	15.4	8.2	32 (0.8414)	Yes	71
Q5	34.8	13.1	51 (0.8989)	Yes	73
Total	336.2	215.1			

Discussion

The effect of aging on the microsomal content of the six chaperones showed markedly different patterns (Figs. 6–13; Table 1). The most unusual patterns were seen with Q5 (Fig. 6; Table 1), Q2 (Fig. 8; Table 1), and BiP (appendix Fig. 11; Table 1). The constitutive levels of Q5 were highest in the youngest animals and showed a 51% decline as the animals reached maturity (Dotted line, Fig. 6). This

pattern would be predicted for a constituent that is critical for growth and proliferation. Since on reaching maturity the hepatocytes enter into a G_0 phase, these cells have far less demand for new membrane components. Hence, it would be expected, as observed, that a chaperon involved in membrane synthesis would show significant decreases. After the age of 200 days the Q5 content showed a slower, but steady, decline. Q5 also showed a marked seasonal variation, having peaks that coincided with midwinter and midsummer. (Fig. 6). This is illustrated more clearly when the constitutive levels are subtracted and the remainder replotted (Fig. 7).

The rhythm coincides with the most stressful seasons of the year. Thus, cyclic variation indicates "stress responsiveness" of the respective chaperone. This stress arose from humidity changes, which range between 30 and 70% during the year. The initial peak in the first winter is markedly blunted compared to the following summer, resulting from the very high constitutive levels observed until the age of 200 days (Fig. 6). Hence, the cells were already producing almost maximal quantities of the respective chaperon. The constitutive levels declined in later ages, but the animals still showed a marked stress response (Fig. 7). As has been suggested by other studies, the stress response decreased with age, showing a 73% decline by 874 days.

Q2 showed a pattern similar to Q5, except for constitutive levels. Q2 content showed increasing constitutive levels as the animals reached maturity and then showed a 32% decline between the ages of 84 days and 874 days. (Fig. 8). The levels also showed a stress response. When the constitutive levels are subtracted out, the levels have a circum semiannual rhythm. These peaks also correspond to midwinter and midsummer. (Fig. 9). This stress response showed a 71% decline with age.

The changes in the ratio of the constitutive levels of Q2 to Q5 corresponding to age is important. Q5 catalyzes the configuration of nascent and denatured proteins into their native tertiary structure and the formation of disulfide bonds. Additionally, although it is not a transferase, Q5 also is required for the N-glycosylation of membrane and secretory proteins. Recent studies indicated that Q2 is involved in the insertion of N-glycosylated proteins into the membrane. The peak demand for the insertion of N-glycosylated proteins into the membrane is at an early age when the animals are rapidly growing. After the liver reaches its mature weight, the cell would have much less need under constant environmental conditions for high levels of proteins that help with insertion because the cells are no longer

growing or proliferating. Thus, Q2 is not the primary chaperon in the hepatocyte, at least in young animals.

Unlike Q2 and Q5, ERp72 showed no seasonal variation. However, similar to Q5, the young animals had the highest concentrations and showed a 30% decline with age. (Fig. 10; Table 1). Although the exact function of ERp72 has not been clearly defined, it is known to be a chaperon and is one of the most highly conserved proteins in the animal kingdom. Human ERp72 reportedly is identical to the nematode, *Caenorhabditis elegans*. Any such highly conserved protein likely serves a critical role in cellular metabolism. Presumably, this relates to its chaperon activity.

BiP is a member of the HSP 70 family believed to serve primarily as a scavenger of improperly edited proteins. BiP showed a pattern similar to Q2 and Q5 (Fig. 11), having a 37% decline with age and some seasonal variation.

Unlike all of the above-described chaperones, calreticulin (a chaperone critical for cell function) showed no significant decline with age. (Fig. 12). Yet its membrane bound homolog, calnexin, showed a marked decline but no seasonal variation (Fig. 13). Furthermore, like Q2, calnexin had low concentrations in young animals, reached a peak at 84 days, and then declined 32% by the age of 874 days. The role of this chaperon in protein synthesis has been extensively studied and is critical to the synthesis of both membrane and secretory proteins.

Conclusion

Since the above data are reported per milligram microsomal protein, it is clear that all of them, with the exception of calreticulin, showed marked, statistically significant declines in specific content with age (30–50%) ($R^2 = 0.9586$ to 0.8414). (i.e. these proteins were declining out of proportion to the other proteins in the hepatic ER). Furthermore, this decline occurred in an organ, the liver, whose function is thought to remain relatively well-conserved with age. Unlike the CNS, immune system, endocrine organs, or kidneys, the liver neither loses weight nor overtly loses of function. For example, the serum levels of the primary secretory protein produced by the liver, albumin, does not decline with age, in spite of a clear loss of protein synthetic capacity.

Example 2 – – Identification of a Chaperone in Human Cerebrospinal Fluid

Chaperones form soluble complexes with a wide variety of secretory proteins. Since the animal study of chaperones in the hepatic microsomes showed a

decline in level of some chaperones as the animals aged, it is important to determine whether any of these chaperones are associated with human cerebrospinal fluid and whether they play a role in Alzheimer's disease.

Materials and Methods

Cerebrospinal fluid samples were clinical waste provided by the Anesthesia Service of the VAMC and the Laboratory Service, Regions Medical Center, St. Paul, MN from normal, healthy patients who were subjected to a spinal tap for spinal anesthesia or to test for meningitis. The patients from whom cerebrospinal fluid samples were taken included 3 infants, 1 young adult, and 4 elderly patients.

The cerebrospinal fluid was examined for the presence of six chaperones: ERp72, calnexin, calreticulin, BiP, Q2, and thiol:protein disulfide oxidoreductase of the form Q5 (Q5). ERp72, calreticulin, calnexin Q2, and Q5 were prepared by methods known in the art. Srivastava et al., J. Biol. Chem., **265**: 8392–99 (1991); Chen et al., Biochemistry, **25**: 8299–8306 (1996).

The chaperone content was determined by immunoblotting the preparations according to the methods described in Example 1. The concentration of chaperone was determined by comparison of various concentrations to the average density of three channels on each gel containing a reference sample of cerebrospinal fluid. The cerebrospinal fluid standards were calibrated against several concentrations of the standard cerebrospinal fluid. The determinations of the proteins in the cerebrospinal fluid were all within the linear range of the immunoassays. Polyclonal antibodies to all chaperones were developed or obtained according to the methods of Example 1.

The immunoblot was also incubated with antibodies to β–amyloid 1–42 (AMY–33; Zymed, South San Francisco, CA).

Results

Only Q2 was identified in human cerebrospinal fluid. The levels of other chaperones, if present, were undetectable. Q2 showed a diffuse band corresponding to a molecular weight of approximately 62 kDa (Fig. 14A, lanes 3–7 and 9–14). Human cerebrospinal fluid was compared to rat hepatic microsomes. The rat microsomes showed a sharp band corresponding to a molecular weight of approximately 57 kDa (Fig. 14A, lane 2). Similarly, purified Q2 showed a sharp band corresponding to a molecular weight of 57 kDa (Fig. 14A, lane 8). The identity of Q2 was confirmed with rabbit monoclonal antibodies to Q2 (GRP58, StressGen, Vancouver, B.C.) (not shown).

The antibodies to β–amyloid 1–42 reacted with rat hepatic microsomes (Fig. 14B, lane 2), but they did not react with purified Q2 (Fig. 14B, lane 8).

Discussion

Because the reaction of β–amyloid 1–42 antibodies with rat, hepatic microsomes and the reaction of antibodies to Q2 with rat hepatic microsomes showed similar bands at molecular weights that correspond to about 57 kDa, this suggests β–amyloid and Q2 form a complex in the liver. Moreover, because β–amyloid 1–42 antibodies did not react with purified Q2, the results do not arise from cross reactivity.

Although the immunoblot incubated with Q2 antibodies and the immunoblot incubated with β–amyloid 1–42 antibodies showed different patterns, the patterns were still similar. This would suggest that the two are present in cerebrospinal fluid as a tight complex.

Conclusion

The chaperone Q2 is present in human cerebrospinal fluid.

Example 3 – – Isolation of a Complex of Q2 with β–amyloid from Human Cerebrospinal Fluid

A complex of Q2 and β–amyloid was discovered and isolated from human cerebrospinal fluid, leading to a better understanding of the role of Q2 in human cerebrospinal fluid and Alzheimer's disease.

Materials and Methods

An immunoblot was prepared according to Example 1 to examine the 62 kD band observed in human cerebrospinal fluid. The immunoblot was incubated with a polyclonal antibody to Q2 and a second immunoblot was incubated with monoclonal antibodies to β–amyloid 1–42 (AMY–33 from Zymed, South San Francisco, CA). A diffuse band was identified again at 62 kDa for cerebrospinal fluid. The band for the monomeric form of β–amyloid 1–42 corresponded to a molecular weight of 5.5 kDa.

Q2: β–amyloid complex was isolated by affinity chromatography using chicken–anti–β–amyloid antibody. Alternatively, isolation by affinity chromatography was accomplished with anti–Q2. The complex was identified by

immunoblotting as described in Example 1. The complex was further purified by chromatography on a Sephacryl column followed by a monoQ column.

Polyclonal antibodies to a purified sample of Q2 were developed in laying hens as described in Example 1. Synthetic β–amyloid 1–42 antibodies also were prepared in chickens by the same method.

Cerebrospinal fluid samples eluted from affinity chromatography with chicken–anti–β–amyloid and, alternatively, from affinity chromatography with anti–Q2, were examined by immunoblot. The chicken antibodies to Q2 and β–amyloid were bound to CNBr–activated Sepharose. The cerebrospinal fluid sample was placed on the column in NaCl (1M). The column was washed and the protein eluted with glycine (.1M, pH 9.0) in NaCl.

Immunoblots were performed after SDS–PAGE and transblotting onto PVDF membranes. Srivastava, J. Biol. Chem., **266**: 20337–20344 (1991); Chen et al., Biochemistry, **25**: 8299–8306 (1996); Towbin et al., Proc. Natl. Acad. Sci. USA, **76**: 4350–54 (1979). The samples were heated to 55° C for 5 minutes in the presence of mercaptoethanol. If they were heated to 90° C, the complex aggregated and no bands were observed. The bands were reacted with the appropriate goat anti–immunoglobulin antibody coupled to alkaline phosphatase. The indicator dye was a combination of nitroblue tetrazolium and 5–bromo–4–chloro–3–indoyl phosphate (BioRad, Richmond, CA). The band intensity was quantitated by computer scanning and analysis (NIH Image Version 1.60).

Results

Blots incubated with antibodies to Q2 showed diffuse bands corresponding to a molecular weight of approximately 62 kDa (Fig. 15A, lanes 3 and 4). Similar bands also were detected when blots were incubated with antibodies to β–amyloid 1–42 (Fig. 15B, lanes 3 and 4). Additionally, the anti–β–amyloid 1–42 was shown not to be reactive to Q2.

Blots incubated with antibodies to β–amyloid 1–42 also revealed a band corresponding to a molecular weight of approximately 62 kDa. But β–amyloid has a molecular of about 5.5 kDa. In addition to the 62 kDa band identified on the immunoblots, a 27 kDa band was identified when incubated with anti–β–amyloid 1–42 (Fig. 15B, lane 3). This 27 kDa band was also observed when synthetic β–

amyloid 1–42 was stored at –20° for several weeks and then run on a gel and, therefore, represents a β–amyloid 1–42 pentamer.

Monoclonal β–amyloid antibody (AMY–33) is highly specific for β–amyloid and is known to bind only to bind to fibrinogen outside of β–amyloid. Stern et al., FEBS Let., **245**: 43–47 (1990). But the molecular weight bands observed in this study were different from those observed with fibrinogen. Moreover, anti–fibrinogen antibodies did not react with any protein in cerebrospinal fluid.

Scanning the blots indicated that 95% of the immunoreactive β–amyloid was associated with the 62 kDa band, whereas 5% was associated with bands of lower molecular weight. This corresponds to a high binding affinity.

These results were confirmed in 100 additional cerebrospinal fluid samples. The same pattern of immunoreactive bands to Q2 and β–amyloid were observed for all 100 samples.

Discussion

Most groups that have examined the effect of Alzheimer's disease on the cerebrospinal fluid concentrations of β–amyloid have used a variety of ELISAs or similar assays, but five have performed immunoblot studies and each observed markedly different results. One study observed only low Mr immunoreactive bands. Ida et al., J. Biol. Chem., **271**: 22908–22914 (1996). Yet the present study showed that 95% of the immunoreactive β–amyloid 1–42 corresponded to a molecular weight of 62 kDa and only 5% with the lower molecular weight. The other groups apparently used more highly denaturing conditions in the preparation of their samples than those used in this study, presumably dissociating the complex. In the present study the complex was not dissociated.

Other studies may have observed different immunoreactive bands because the antibodies may have differed in specificity from those used here. Seubert et al., Nature, **361**: 260–63 (1993); Globek et al., Neurosci. Let., **191**: 79–82 (1995).

In a study of the interaction of β–amyloid with high density lipoproteins, an immunoreactive band at 62 kDa was observed (Koudinov et al, Biochem. Biophys. Res. Commun., **223**: 592–97 (1996); Ghiso et al., Biochem. J., **293**: 27–30 (1993)), but soluble β–amyloid from the fraction that produced a 62 kDa band was not retrieved and the fraction that produced the 62 kDa band was not identified or characterized.

The band corresponding to a molecular weight of 62 kDa would be indicative of a complex between β–amyloid 1–42 and Q2. Because this band was diffuse, this suggests that the complex between β–amyloid 1–42 and Q2 is glycosylated.

Conclusion

A complex of Q2 and β–amyloid 1–42 was found in human cerebrospinal fluid. By binding to β–amyloid 1–42, Q2 keeps β–amyloid 1–42 in solution and helps prevent aggregation and precipitation of β–amyloid.

Example 4 – – Characterization of a Complex of Q2 with β–amyloid 1–42

The complex of Q2 and β–amyloid was characterized to better understand the role of this complex in preventing formation of β–amyloid plaques and in Alzheimer's disease.

Materials and Methods

A complex of Q2 with β–amyloid was purified as described in Example 3.

The presence of carbohydrate moieties on the complex was determined by methods known to those of skill in the art. In particular, the complex was reacted with PAS. The PAS–reacted complex was eluted through a boronate column.

Results

When the complex was reacted with PAS, a band corresponding to a molecular weight of 62 kDa was identified. Moreover, the PAS–reacted complex bound to a boronate column, which suggests the presence of a carbohydrate moiety.

Discussion

The results from the reaction of the complex with PAS are consistent with the observation of the diffuse band corresponding to a molecular weight of 62 kDa described in Example 3. These results are also consistent with other studies that have indicated that Q2 has lectin–like properties and that Q2 may bind to proteins only after being N–glycosylated. Oliver et al., Science, **275**: 86–88 (1997); Elliott et al., J. Biol. Chem., **272**: 13849–13855 (1997). N–glycosylation may be important in the post–translational processing of many proteins that are synthesized in the endoplasmic reticulum. Suzuki et al., J. Biol. Chem., **272**: 10083–10086 (1998).

The likelihood that the complex is N–glycosylated is also supported by the observation that the complex was relatively stable under the moderately severe

denaturing conditions that were used to solubilize the samples for the SDS–Page and immunoblot analyses.

Conclusi n

Although Q2 and β–amyloid have not been shown to be glycosylated when not in complex, these results suggest that Q2: β–amyloid is glycosylated as a complex.

Example 5 – – Detection of β–Amyloid Plaques and Correlation of Plaque Presence to Q2 Levels and/or apoE Genotype

As Q2 levels decline relative to normal Q2 levels, a person can have an increased susceptibility to, for example, β–amyloid aggregation, having Alzheimer's disease, having symptoms associated with Alzheimer's disease, or likelihood of developing Alzheimer's disease. It is desirable to understand this correlation.

Methods and Materials

Cerebrospinal fluid samples and brain samples were taken from autopsy specimens. The specimens were obtained from 21 nuns who participated in a study in which their health status and psychomotor abilities were evaluated and monitored until their deaths. Snowdon, JAMA, **277**: 813–17 (1997). The cerebral spinal fluid samples were frozen at the time of collection and stored at –70°C .

The cerebrospinal fluid samples were examined for the presence of chaperones as described in Example 1 with some modifications. Immunoblots were performed after SDS–PAGE and transblotting onto nitrocellulose membranes. Laemmli, Nature, **227**: 680–85 (1970); Towbin et al., Proc. Natl. Acad. Sci. USA, **76**: 4350–54(1979). The samples were heated to 55°C for 5 minutes in the presence of SDS and mercaptoethanol. If they were heated to 90°C, the complexes aggregated and no bands were observed. In a first study, the bands were reacted with antibodies specific for the molecule of interest and then with the appropriate goat anti–immunoglobulin antibodies coupled to alkaline phosphatase. The antibodies specific for the molecule of interest were polyclonal antibodies to calmodulin, calnexin, BiP, ERp72, Q2, Q5, and synthetic β–amyloid 1–42. Antibodies to BiP and Q2 were purchased from StressGen (Vancouver, B.C.). Antibodies to β–amyloid 1–42 were purchased from Zymed (AMY–33; South San Francisco, CA). All other antibodies were prepared in laying hens as described in Example 1. The

indicator dye used was a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BioRad, Richmond, CA).

In a second study, cerebrospinal fluid samples eluted from affinity chromatography with anti-Q2 and, alternatively, anti-β-amyloid 1-42, were examined by immunoblot. The antibodies were first bound to CNBr-activated Sepharose. The resin was suspended in NaCl (1M), mixed with 1 ml CSF sample, incubated overnight at 4°C, and then poured into a column. The column was washed with NaCl until the eluate had no absorbance at 280nm. The bound proteins were eluted with glycine (0.1 M, pH 9.0) in NaCl. Q2 and β-amyloid 1-42 were identified according to the immunoblotting procedure described above.

In a third study, the concentration of Q2 was determined from the immunoblots by enhanced chemiluminescence with a peroxidase reaction (RPN 2106; Amersham Life Science, Piscataway, NJ). The band intensities were quantitated by computer scanning of the chemiluminescence photographic film and analyzed by NIH Image (Version 1.60).

The brain samples obtained from the nuns were examined to determine the total number of β-amyloid plaques, including both diffuse and neuritic types of plaque. The total plaque numbers were determined from the five most severely affected microscopic fields of Bielschoasky-stained sections of the frontal, temporal, and parietal lobes of the neocortex.

The brain samples obtained from the nuns were also examined to determine the apoE genotype of the subject. Snowdon et al., J. Am. Med. Assoc., **277**: 813-17 (1997).

Results

The chemiluminescence assay was linear with concentrations of Q2 between about 5 and 45 ng/ml of cerebrospinal fluid.

Figure 16 and Table 2 show a significant correlation between the concentration of Q2 and the total number of plaques ($r = -0.52$, $p < 0.02$, 95% CI -0.25 to -0.79 by the Spearman rank correlation test).

Discussion

The cerebrospinal fluid samples from the nuns showed three distinct groups. The first had an abundant number of plaques and less than 17 ng/ml of Q2 (6 of 21). The second group had normal levels of Q2 and an abundant number of plaques (8 of

21). The third group had little or no plaques and normal levels of Q2. But the third group is the only group that showed only $apoE_2$ or $apoE_3$ alleles (Table 2).

TABLE 2: Ventricular Cerebrospinal Fluid Q2 Concentrations, Senile Plaque Scores, Apolipoprotein E Genotype and Age of the Autopsied Participants in the Nun Study.

Group	CSF Q2 ng/ml	Senile Plaque Scores	ApoE Genotype	Age Years
Low Q2	6.9	21.1	3,3	79
and Abundant	14.3	21.3	3,3	94
Senile Plaque	14.8	21.3	4,4	81
	15.5	21.3	3,3	86
	16.3	21.3	2,3	93
	16.9	21.3	3,3	84
				Average age 86.2 ± 2.3
Normal Q2	17.1	21.3	3,4	89
and Abundant	17.8	21.3	3,4	83
Senile Plaque	23.8	20.8	3,4	88
	25.3	21.3	4,4	85
	26.7	20.8	3,3	91
	29.0	21.3	3,4	80
	32.1	20.8	3,3	96
	33.6	21.0	3,3	92
				Average age 88.0 ± 1.7
Normal Q2	17.8	4.1	3,3	81
and Little or	21.0	4.9	3,3	83
No Senile	22.1	0.0	3,3	82
	22.2	0.0	3,3	86
	25.4	0.0	2,3	92
	31.6	0.0	2,3	97
	31.9	0.0	2,2	90
				Average age 87.3 ± 2.1

As described in Example 3, about 5% of β–amyloid 1–42 is free in solution in normal subjects. Some studies have shown that $apoE_4$ more readily forms insoluble complexes with β–amyloid than do other isoforms of the apolipoprotein. Moir et al., Biochemistry, **38**: 4595–4603 (1999). The results shown in Table 2 suggest that individuals having an $apoE_4$ genotype may form plaques at lower concentrations of free β–amyloid than do the other genotypes.

The data shown in Figure 16 and Table 2 show that a person who does not have $apoE_4$ genotype and has normal Q2 levels has no or few plaques. Yet a person with low levels of Q2 has increased susceptibility to an abundant amount of plaques,

irrespective of apoE genotype. That is, 100% of the people studied who showed a decline in Q2 levels relative to normal had an abundant amount of plaque. A person with normal levels of Q2 but with an $apoE_4$ allele also has increased susceptibility to an abundant amount of plaques. It should be noted, however, that only half of the people having normal levels of Q2 and an abundant amount of plaques had an $apoE_4$ allele. Thus, a third gene or protein may also be involved in the development of Alzheimer's disease.

The specimens from the nuns were also compared to specimens from 12 young people (3 to 16 years of age) and from 12 elderly people without Alzheimer's disease (66 to 82 years of age). The Q2 levels from the young subjects (27.3 ± 1.2 ng/ml) and the elderly subjects (29.9 ± 1.6 ng/ml) were the same as the Q2 levels observed in the nuns who had little or no senile plaques (24.6 ± 1.9 ng/ml).

Conclusion

This study demonstrates an increased incidence of Alzheimer's disease in individuals with at least one $apoE_4$ allele and in individuals with Q2 levels less than 17 ng/ml, independent of $apoE_4$ genotype.

This invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains.

What is claimed is:

1. An isolated complex comprising a Q2 and a β–amyloid.

2. The isolated complex of claim 1, wherein the β–amyloid comprises animal β–amyloid 1–42, animal β–amyloid 1–38, human β–amyloid 1–42, or human β–amyloid 1–38.

3. The isolated complex of claim 1, wherein the complex is glycosylated.

4. A method of detecting in a biological sample a complex of Q2 and β–amyloid, comprising:

obtaining a biological sample; and

conducting an immunoassay or fluorescence polarization assay to recognize a Q2, a β–amyloid, or the complex of Q2 and β–amyloid.

5. The method of claim 4, comprising conducting the immunoassay with one or more antibodies recognizing Q2, β–amyloid, or the complex of Q2 and β–amyloid.

6. The method of claim 4, wherein the biological sample comprises human or animal biological material from a central nervous system.

7. The method of claim 6, wherein the biological sample comprises human or animal cerebrospinal fluid.

8. The method of claim 4, further comprising correlating a level of the complex to a level of β–amyloid aggregation in the biological sample.

9. The method of claim 4, wherein the biological sample comprises human biological material from central nervous system and the method further comprises correlating the level of the complex with a susceptibility to Alzheimer's disease.

10. The method of claim 9, wherein the biological sample comprises human biological material from central nervous system and the method further comprises

correlating the level of the complex with a likelihood of developing Alzheimer's disease.

11. The method of claim 4, wherein the biological sample comprises human biological material from central nervous system and the method further comprises correlating the level of the complex with behavioral change in a human from whom the biological sample was obtained.

12. A method of detecting aggregation of β–amyloid, comprising:

obtaining a biological sample; and

determining a level of Q2 in the biological sample.

13. The method of claim 12, further comprising correlating a decline in the Q2 level with an increase in aggregation of β–amyloid.

14. The method of claim 12, wherein the Q2 is a component of a complex comprising a Q2 and a β–amyloid.

15. The method of claim 14, further comprising correlating a decline in a level of Q2 in a complex comprising Q2 and β–amyloid with an increase in aggregation of β–amyloid.

16. The method of claim 12, wherein β–amyloid comprises animal β–amyloid 1–42, animal β–amyloid 1–38, human β–amyloid 1–42, or human β–amyloid 1–38.

17. The method of claim 12, wherein the biological sample comprises human or animal biological material from central nervous system.

18. The method of claim 17, wherein the biological sample comprises human or animal cerebrospinal fluid.

19. The method of claim 12, wherein detecting aggregation of β–amyloid comprises detecting formation of a β–amyloid plaque.

20. The method of claim 12, wherein detecting aggregation of β–amyloid comprises screening for an increased susceptibility to Alzheimer's disease.

21. The method of claim 12, further comprising screening for apoE genotype.

22. A method of screening for Alzheimer's disease, comprising:
obtaining a human biological sample;
determining a level of Q2; and
correlating the Q2 level to a susceptibility to Alzheimer's disease.

23. The method of claim 22, wherein correlating the Q2 level to the susceptibility to Alzheimer's disease comprises correlating a decline in the Q2 level with an increase in aggregation of β–amyloid.

24. The method of claim 22, wherein Q2 is a component of a complex comprising Q2 and β–amyloid.

25. The method of claim 24, further comprising determining a level of a complex comprising Q2 and β–amyloid.

26. The method of claim 24, wherein the β–amyloid in complex is β–amyloid 1–42 or β–amyloid 1–38.

27. The method of claim 22, wherein the human biological sample comprises biological material from central nervous system.

28. The method of claim 27, wherein the human biological sample comprises biological material from cerebrospinal fluid

29. The method of claim 22, further comprising correlating the level of Q2 to a level of a complex comprising Q2 and β–amyloid and a level of β–amyloid in cerebrospinal fluid.

30. The method of claim 29, wherein the β–amyloid in complex is human β–amyloid 1–42 or human β–amyloid 1–38 and the β–amyloid in cerebrospinal fluid is human β–amyloid 1–42 or human β–amyloid 1–38, respectively.

31. The method of claim 22, further comprising screening for apoE genotype.

32. The method of claim 22, wherein correlating comprises correlating the level of Q2 with a likelihood of developing Alzheimer's disease.

33. A method for treating Alzheimer's disease comprising administering an effective amount of Q2 to a relevant tissue of a subject in need thereof.

34. The method of claim 33, wherein the relevant tissue is a brain.

35. The method of claim 33, wherein the relevant tissue is cerebrospinal fluid.

36. An antibody recognizing a complex comprising a Q2 and a β–amyloid.

37. The antibody of claim 36, wherein the complex comprises human β–amyloid 1–42, human β–amyloid 1–38, animal β–amyloid 1–42, or animal β–amyloid 1–38.

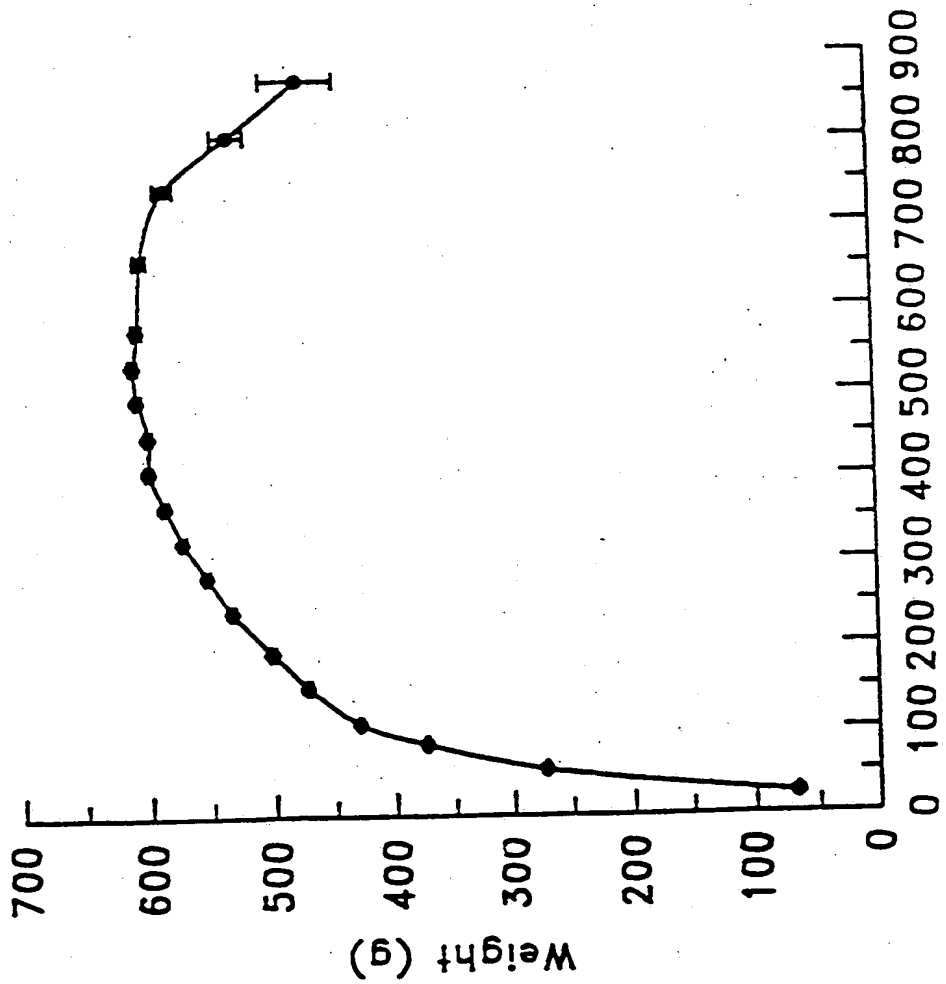
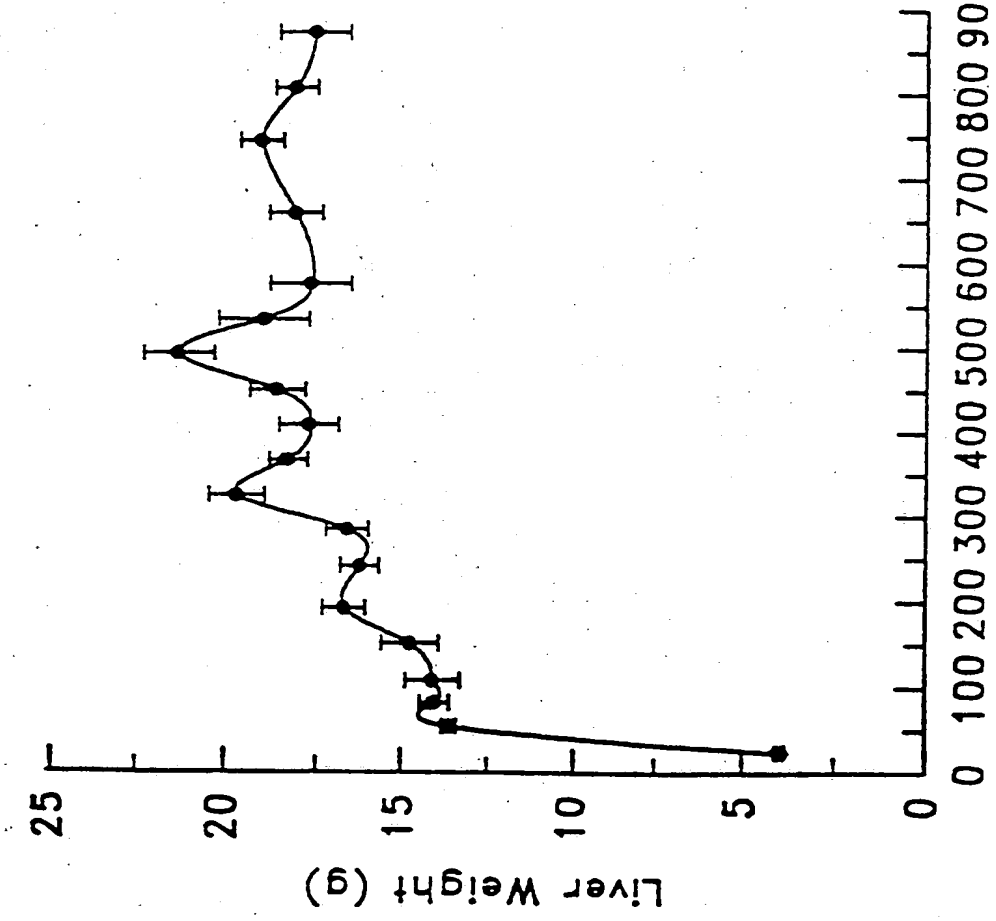


Figure 1: Animal Weights as a Function of Age



10/6/96

Figure 2: Liver Weights as a Function of Age

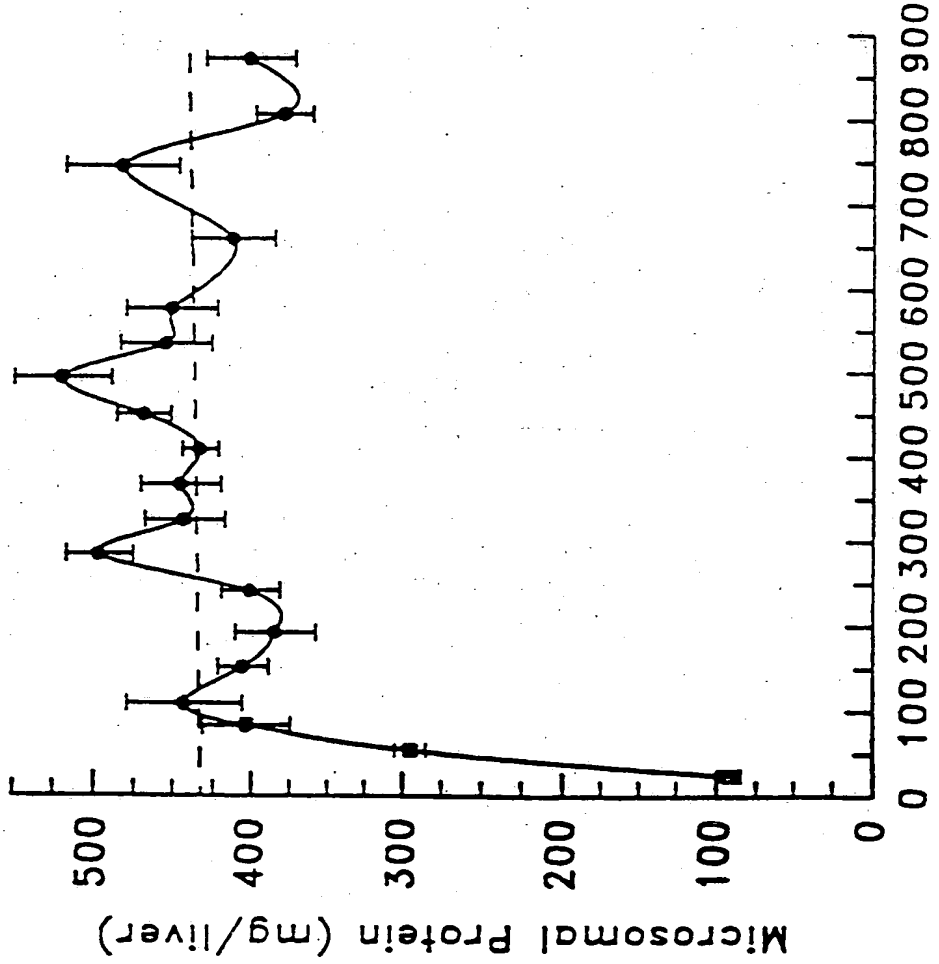


Figure 3: Microsomal Protein per gram Liver as a Function of Age

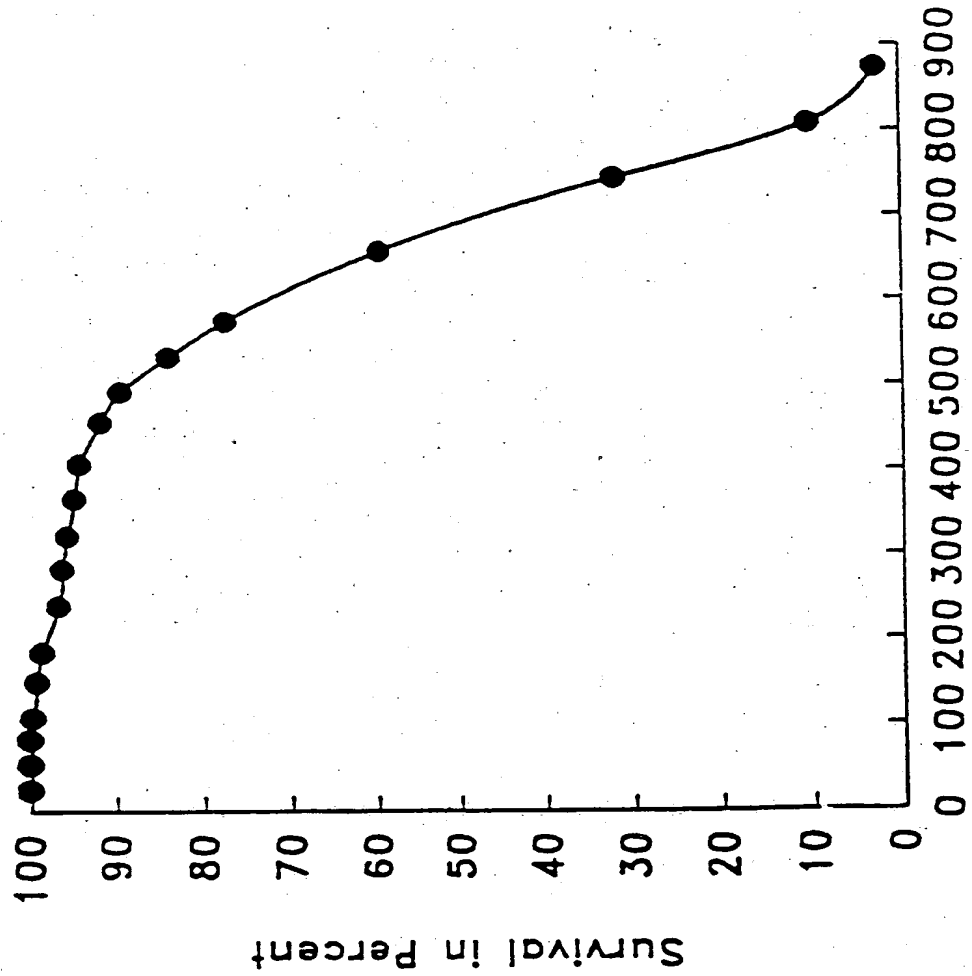


Figure 4: Life Table Analysis of Animal Survival

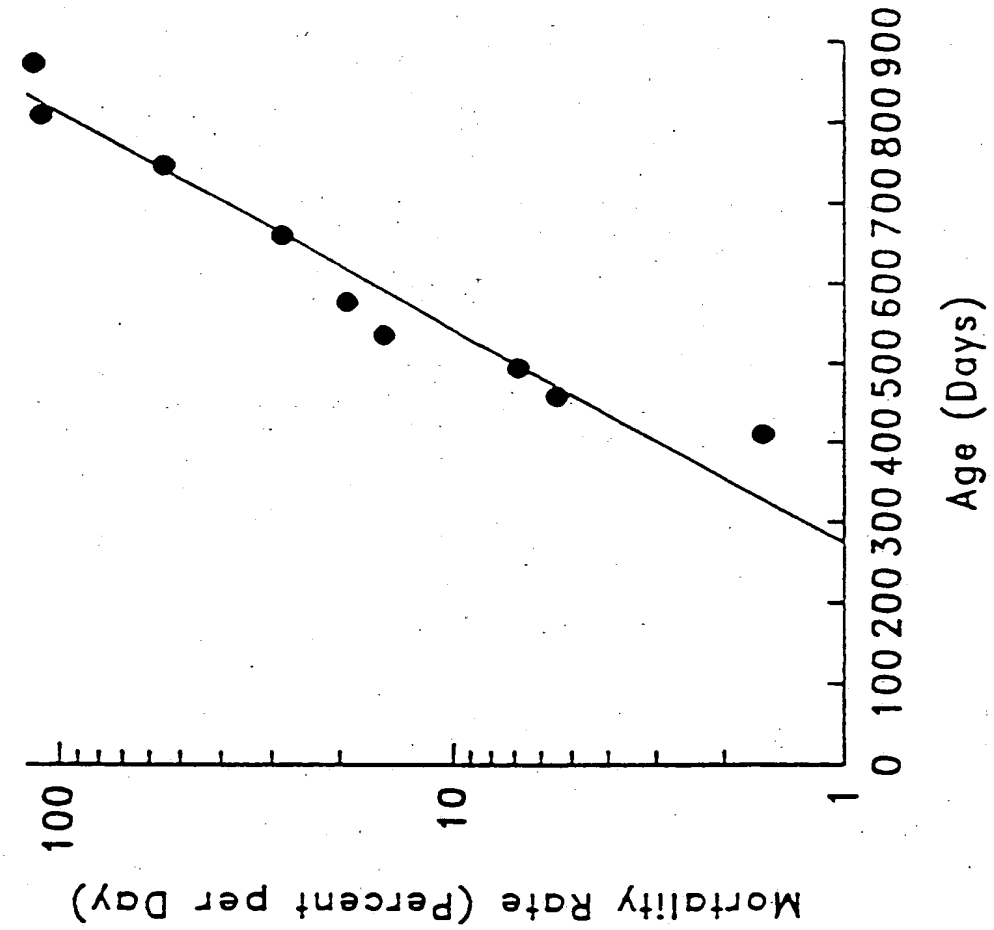


Figure 5: Mortality Rate versus Age

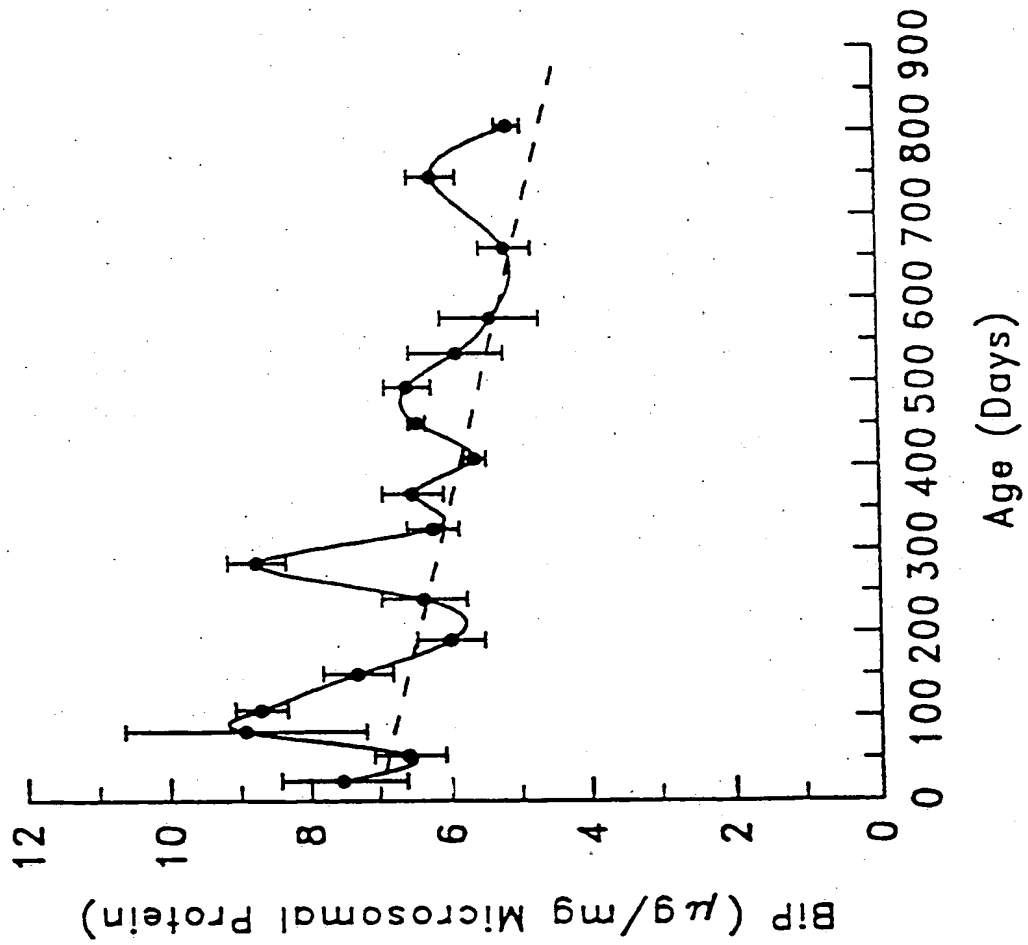


Figure 11: Effect of Age on the Hepatic, Microsomal BiP Levels

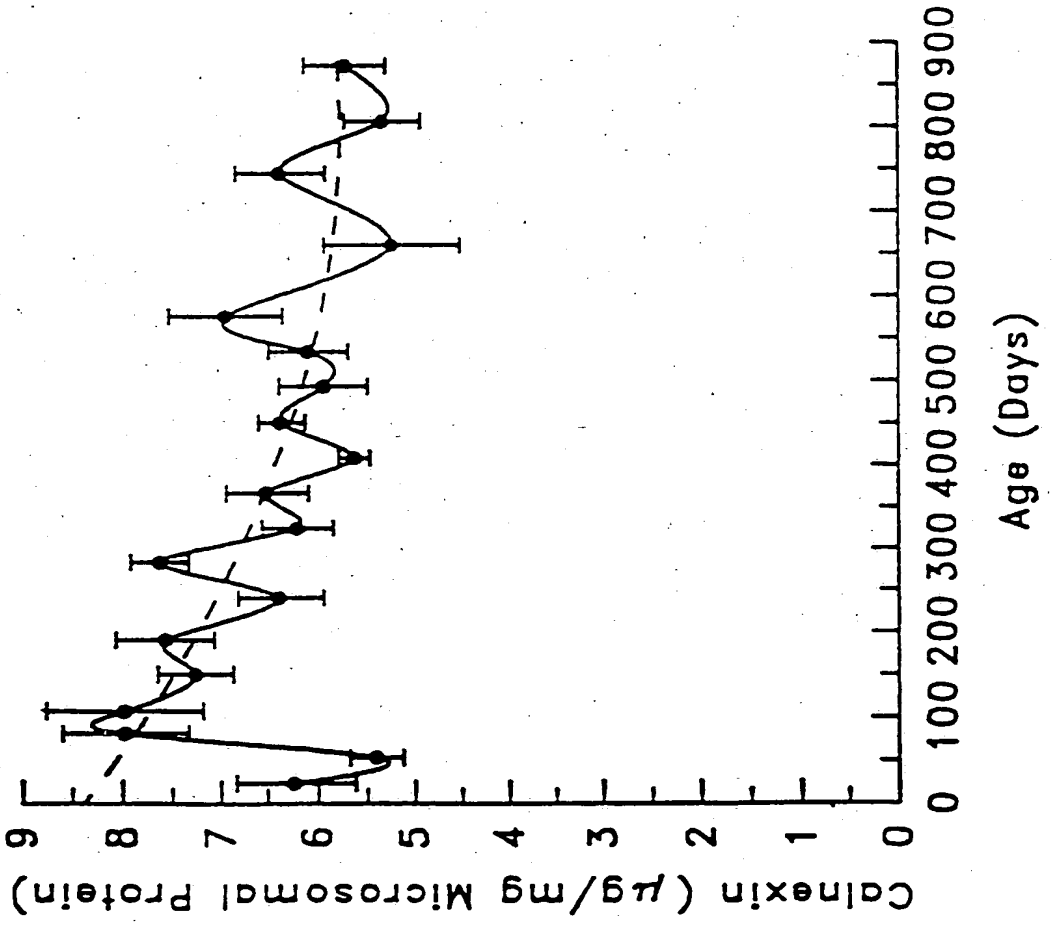


Figure 12: Effect of Age on the Hepatic, Microsomal Calnexin Levels

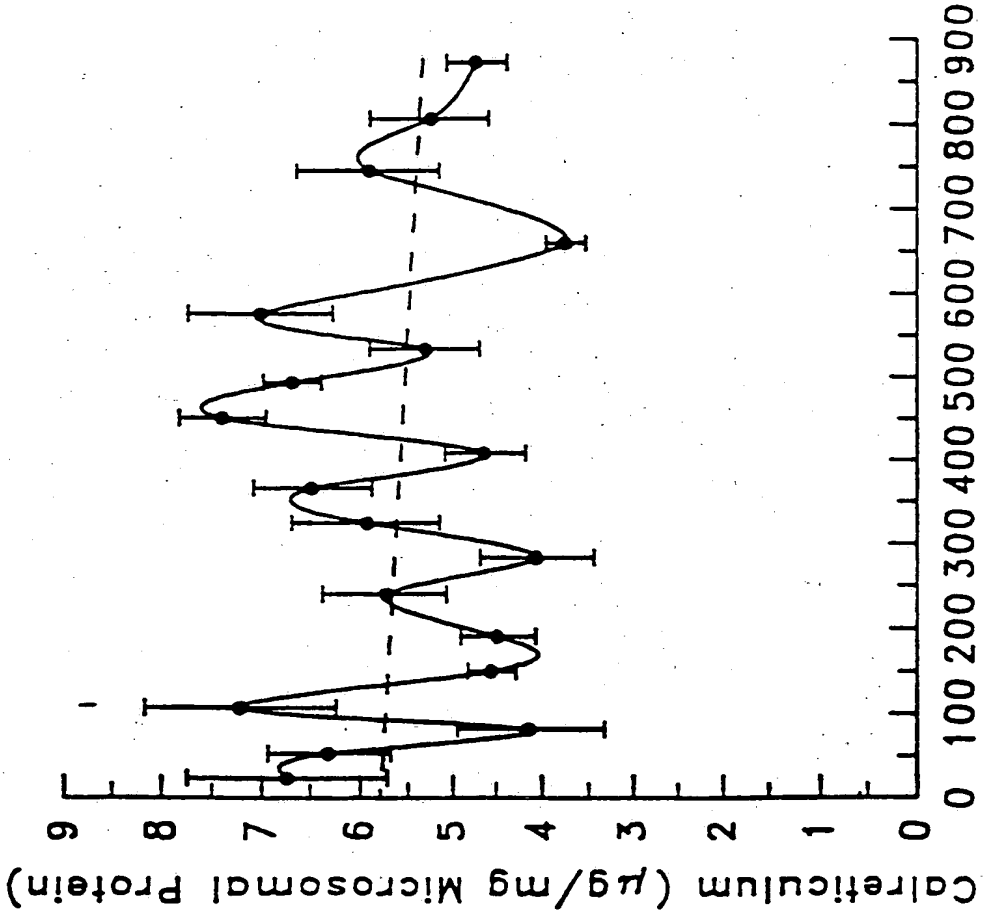
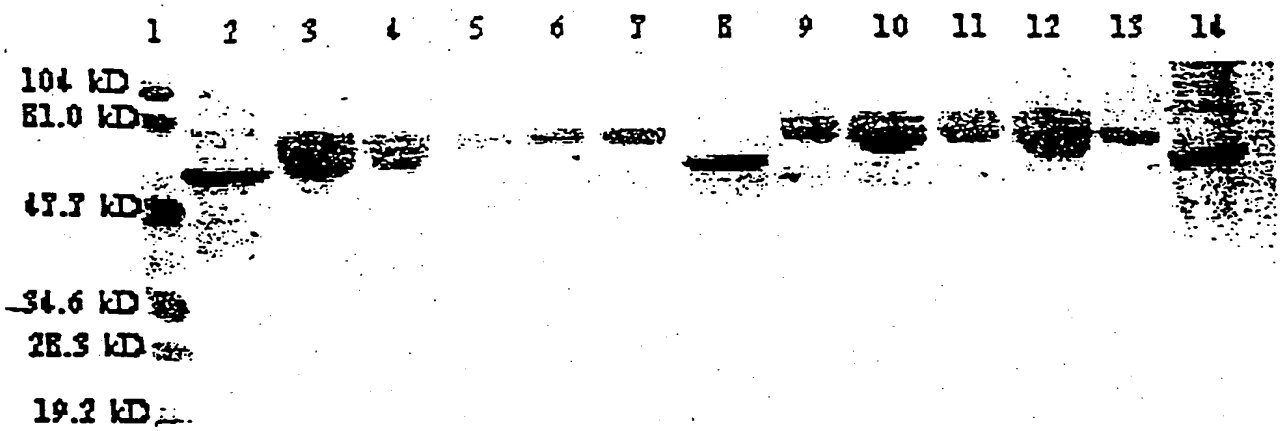


Figure 13: Effect of Age on the Hepatic, Microsomal Calreticulin Levels



B

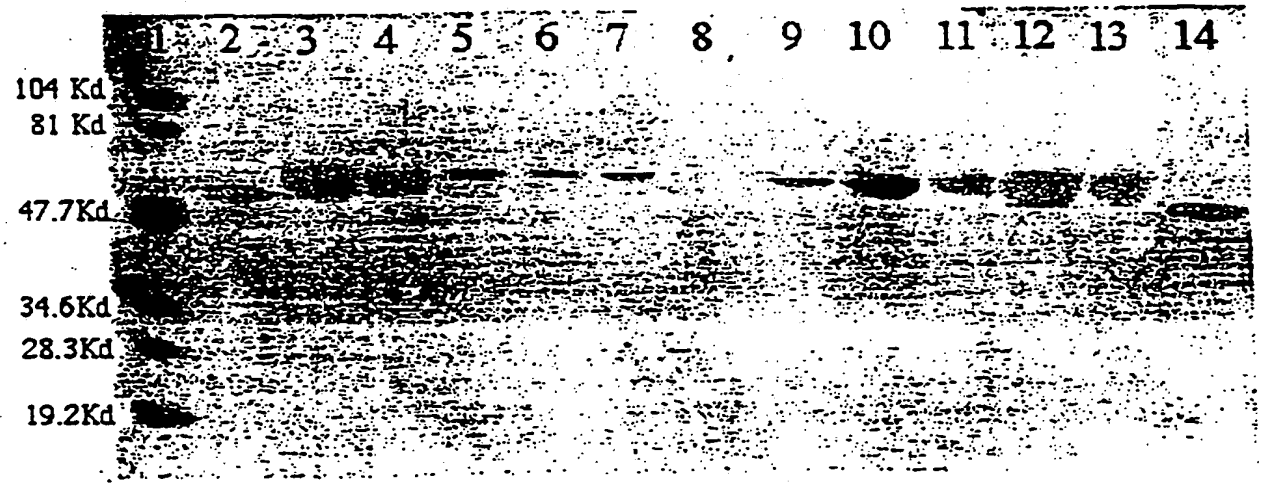


FIGURE 14: Immunoblots with A) Chicken Polyclonal antibodies to Aβ and B) Monoclonal Antibodies to β-amyloid 1-42. Channel 1 - Prestained Standards; Channels 2 - Rat Hepatic Microsomes; Channels 3-7 & 9-14 – 15μl of CSF from Normal, Human Subjects, Ages 6 Months to 59 Years; Channel 8 - Purified Aβ.

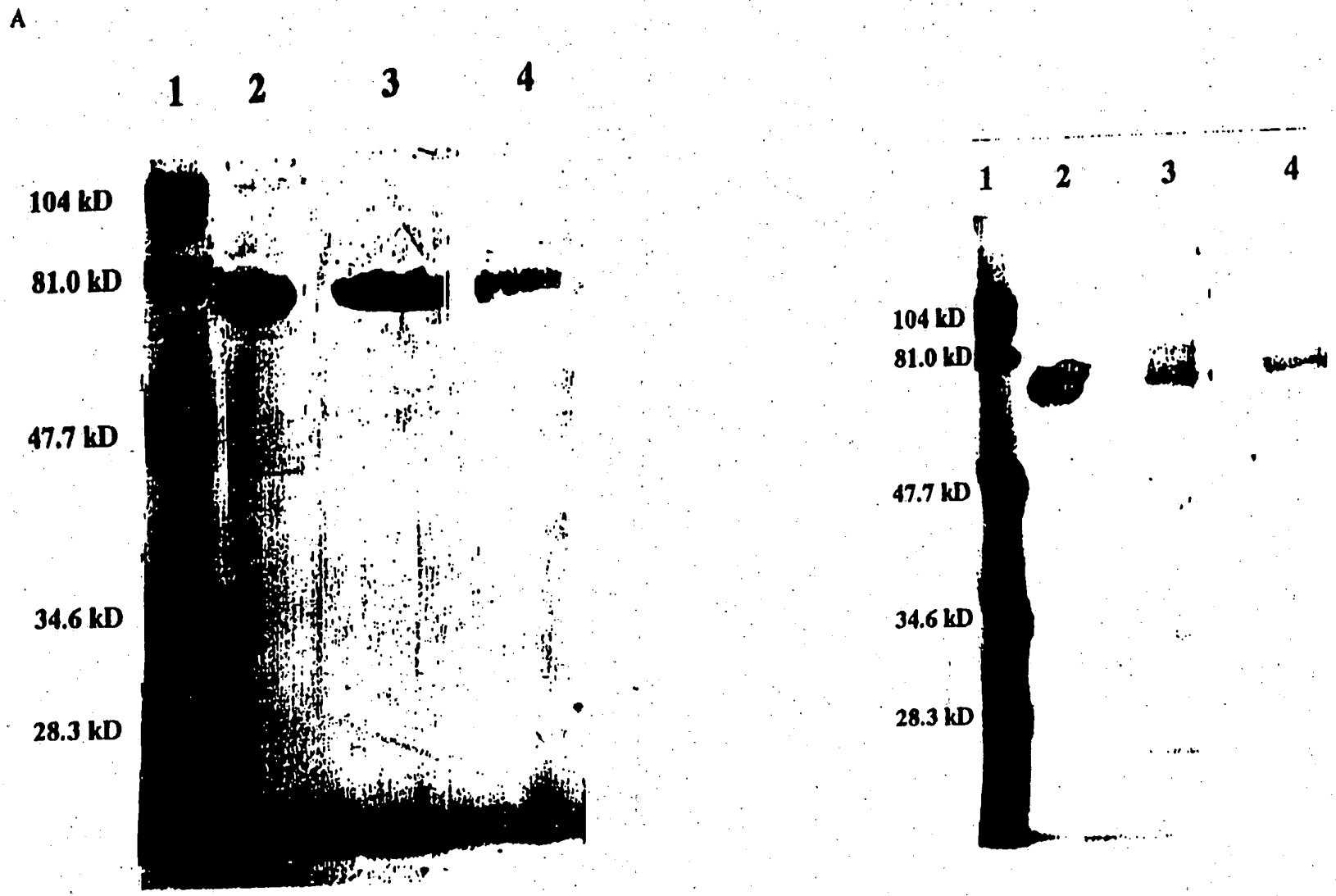


FIGURE 15: Immunoblots with A) Polyclonal antibodies to Q2 and B) to β-amyloid 1-42. Channel 1 - Prestained Standards; Channels 2 - CSF; Channel 3 - CSF Separated by affinity chromatography with anti-β-amyloid antibodies; Channel 4 - CSF Separated by affinity chromatography with anti-Q2 antibodies

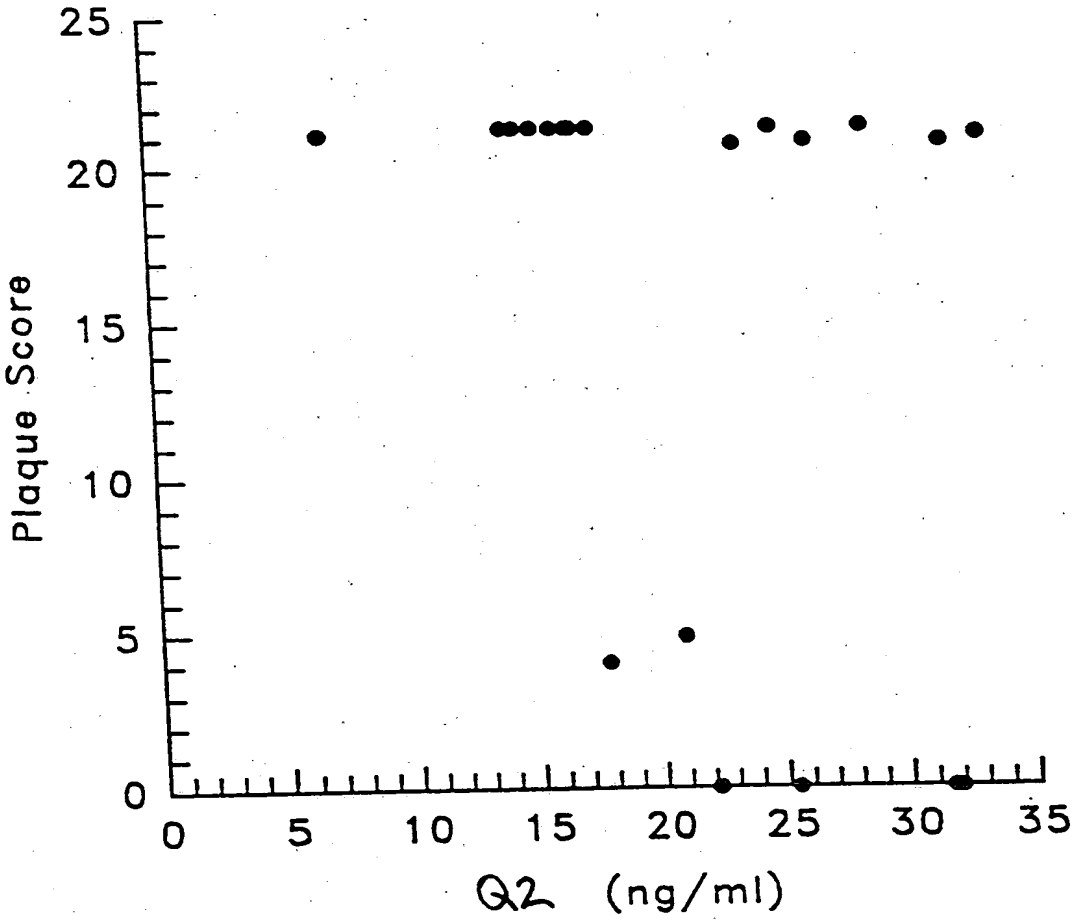


FIGURE 16: The CSF Concentrations of Q2 Compared to Senile Plaque Scores in the Brains of Participants in the Nun Study.

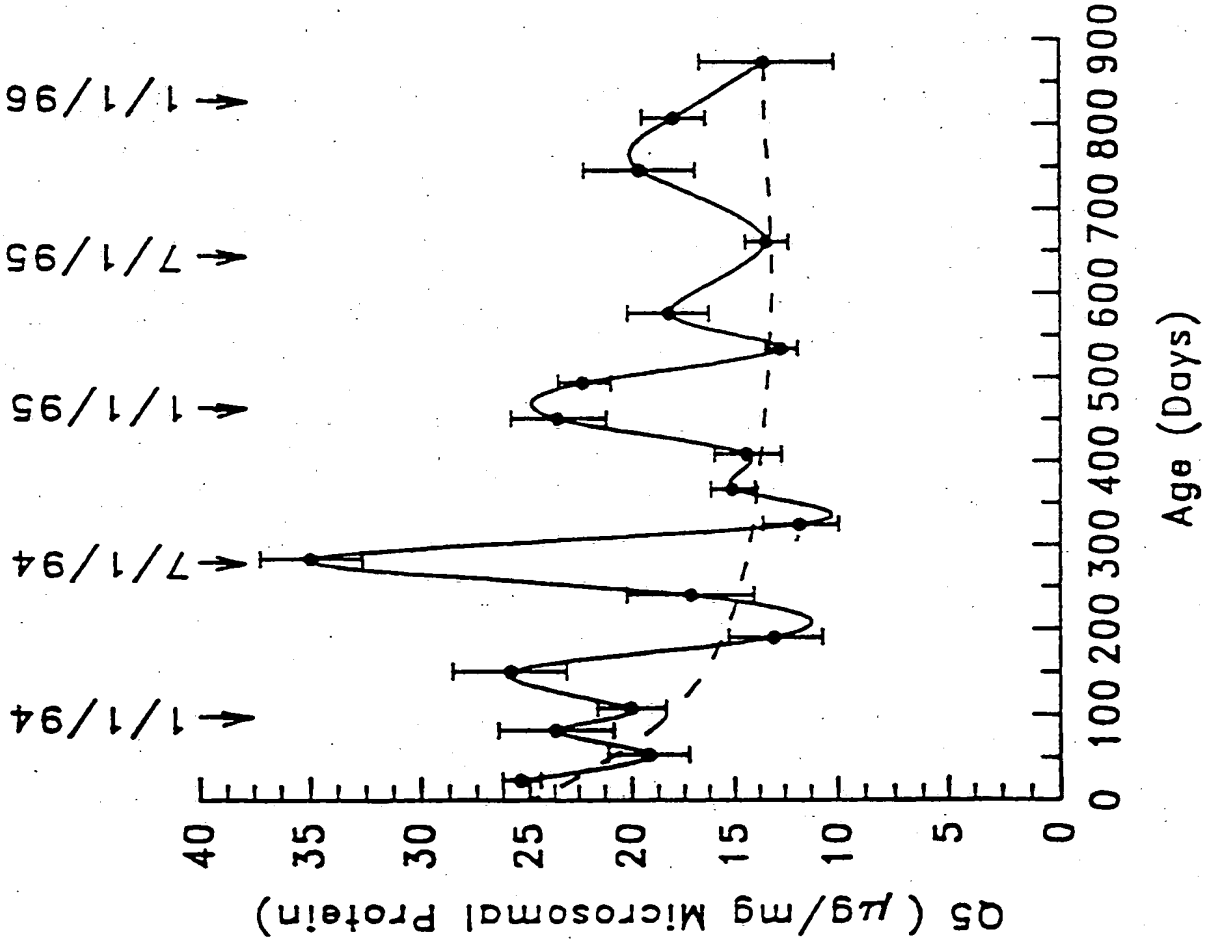


Figure 6: Effect of Age on the Hepatic, Microsomal Q5 Levels

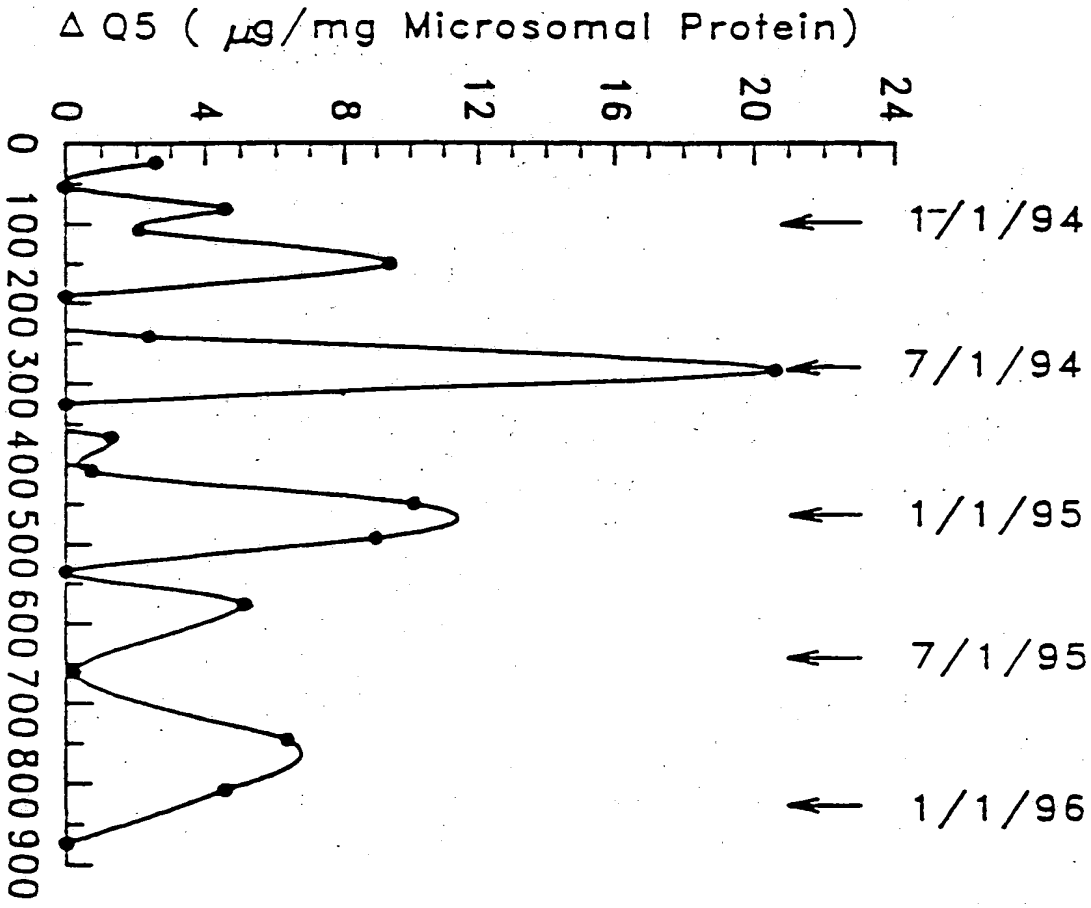


Figure 7: The Effect of Age on the Stress Responsive Levels of Q5.

12/14/96
73% decline between
204 and 746-800 days

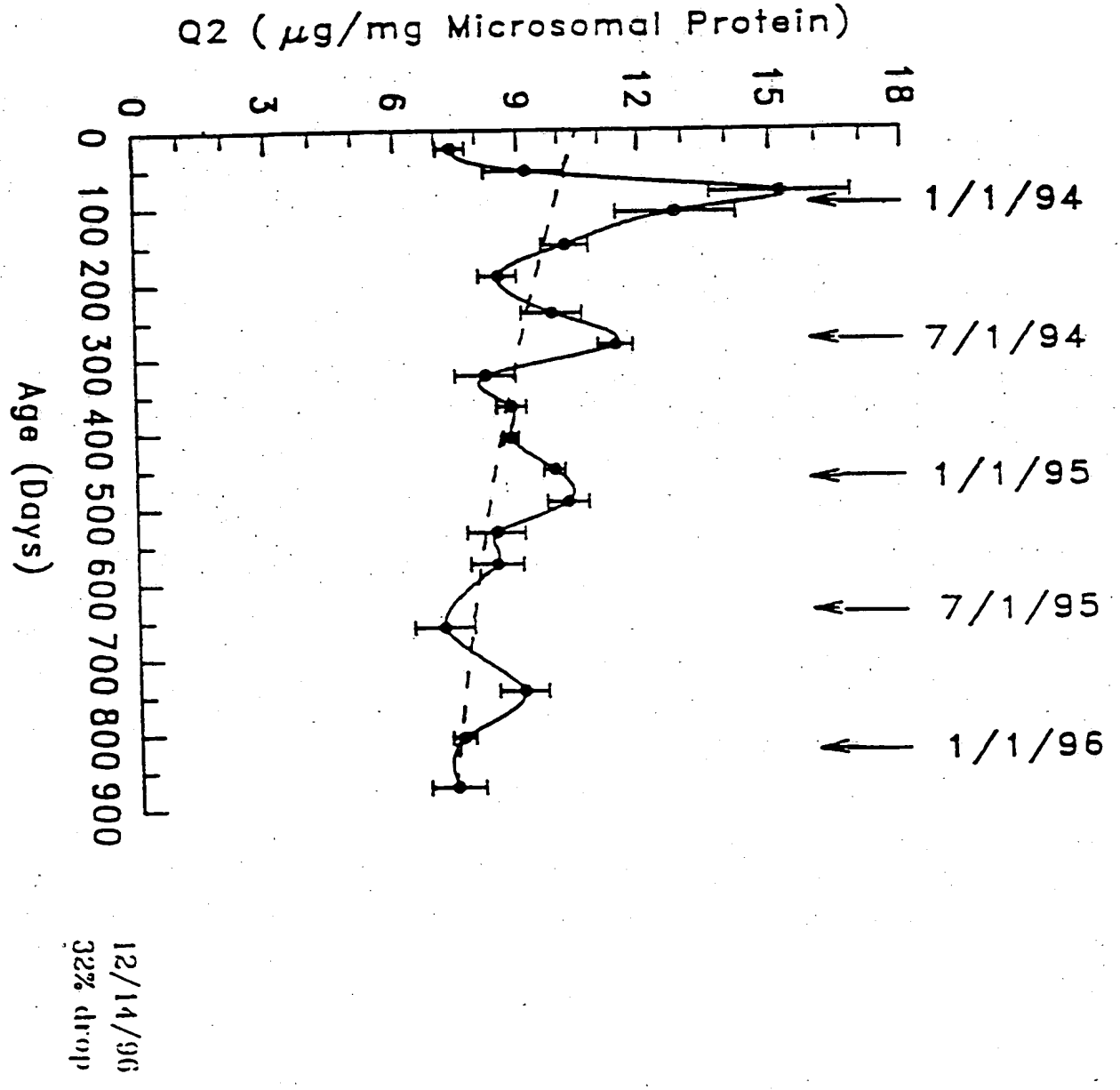


Figure 8: Effect of Age on the Hepatic, Microsomal Q2 Levels

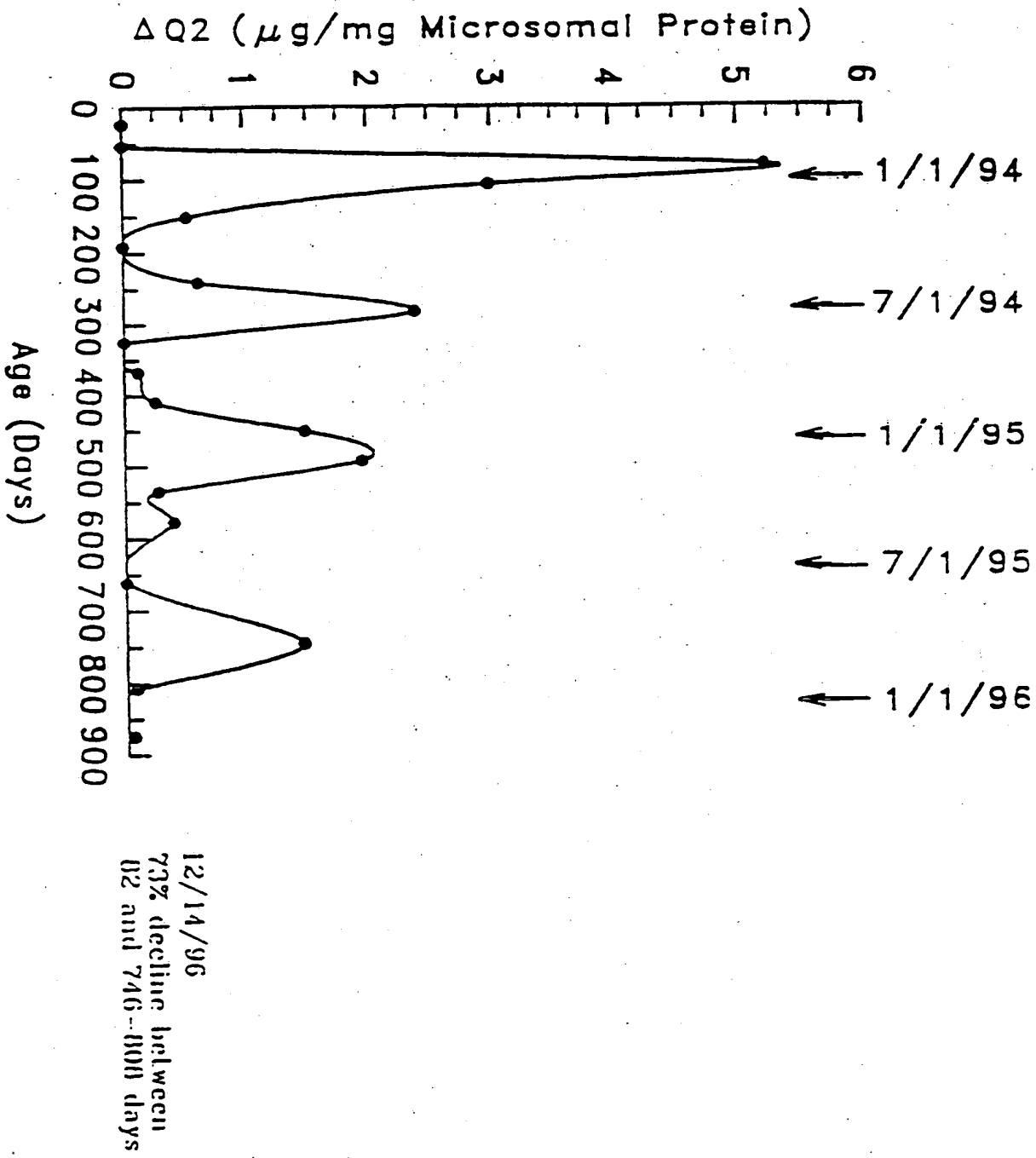


Figure 9: The Effect of Age on the Stress Responsive Levels of Q2.

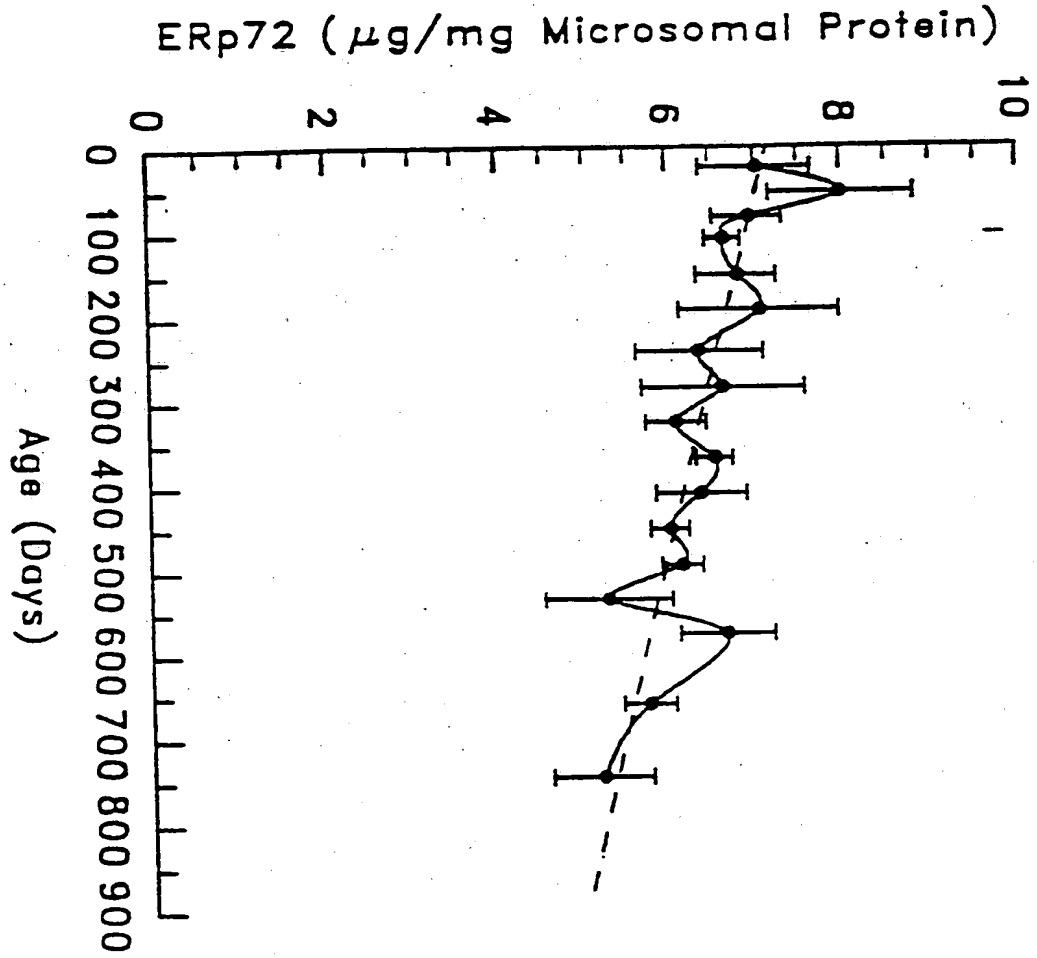


Figure 10: Effect of Age on the Hepatic, Microsomal ERp72 Levels

10/13/96
30% decrease

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 May 2000 (11.05.2000)

PCT

(10) International Publication Number
WO 00/26251 A3

(51) International Patent Classification[7]: G01N 33/68, C07K 14/47

(21) International Application Number: PCT/US99/25593

(22) International Filing Date: 29 October 1999 (29.10.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/106,398 30 October 1998 (30.10.1998) US
60/123,564 10 March 1999 (10.03.1999) US

(71) Applicant and
(72) Inventor: HOLTZMAN, Jordan, L. [US/US]; 4710 Girard Avenue South, Minneapolis, MN 55409 (US).

(74) Agent: DAIGNAULT, Ronald, A.; Merchant & Gould P.C., 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402-4131 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
10 August 2000

(48) Date of publication of this corrected version:
4 January 2001

(15) Information about Correction:
see PCT Gazette No. 01/2001 of 4 January 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/26251 A3

(54) Title: A COMPLEX OF A CHAPERONE WITH ß-AMYLOID AND METHODS EMPLOYING THIS COMPLEX

(57) Abstract: A chaperone protein Q2 and ß-amyloid can form a complex. This complex can be detected in a biological sample, such as, for example, tissues or fluids from a mammal. Q2 levels can also be detected in a biological sample. A method for determining the Q2 level in a biological sample and comparing that level to a normal Q2 level can be used to detect, screen, diagnose, or otherwise determine a person's susceptibility to Alzheimer's disease such as, for example, the presence or absence of Alzheimer's disease, of symptoms of this disease, of factors leading to or associated with this disease, of likelihood of developing this disease, and the like. In one embodiment, a decline in Q2 level correlates to an increased likelihood of developing Alzheimer's disease. In another embodiment, a decline in Q2 level correlates to an increase in ß-amyloid aggregation. The method may further include screening for an apolipoprotein E genetype, which is associated with Alzheimer's disease.

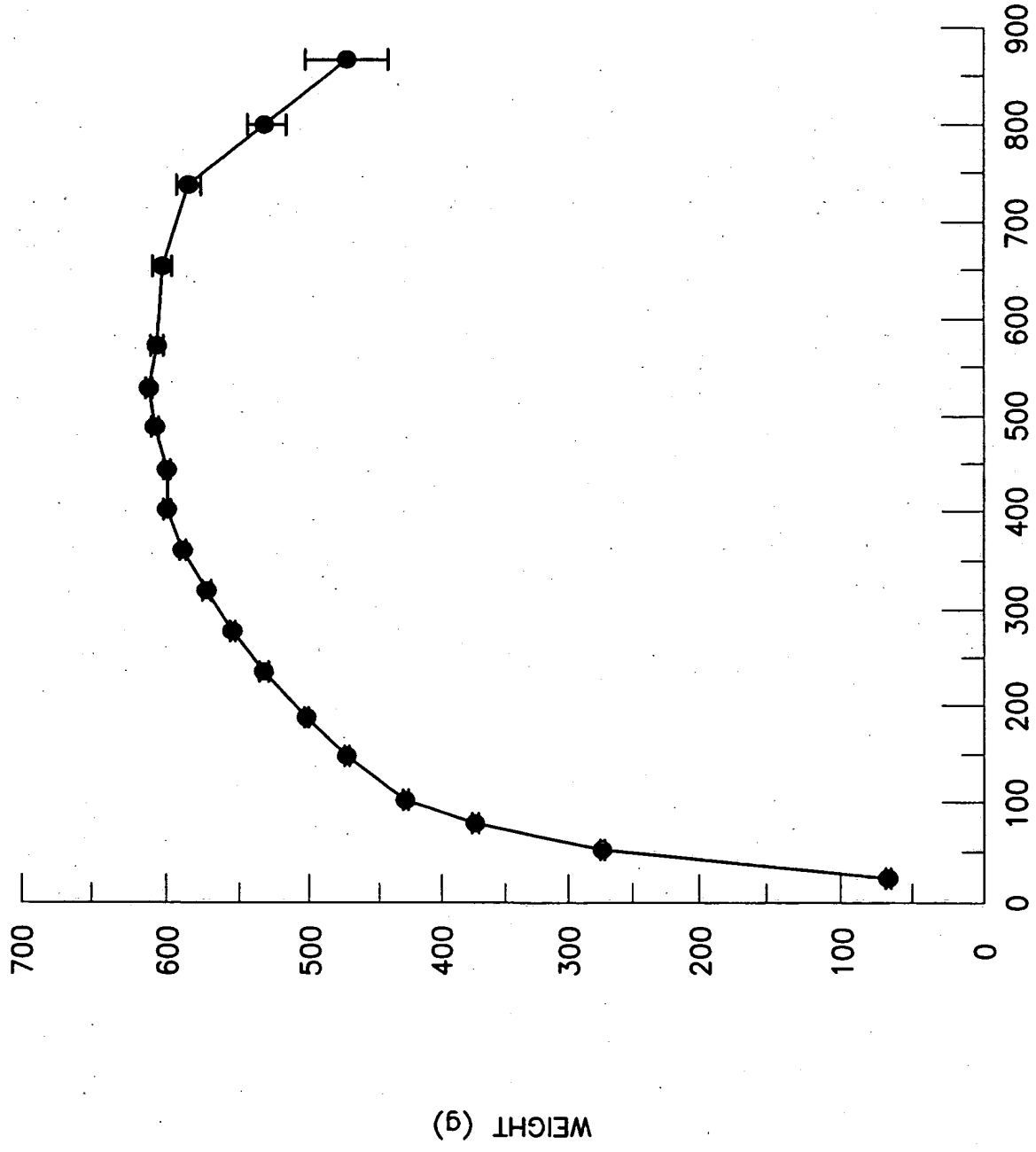


FIG. 1: ANIMAL WEIGHTS AS A FUNCTION OF AGE

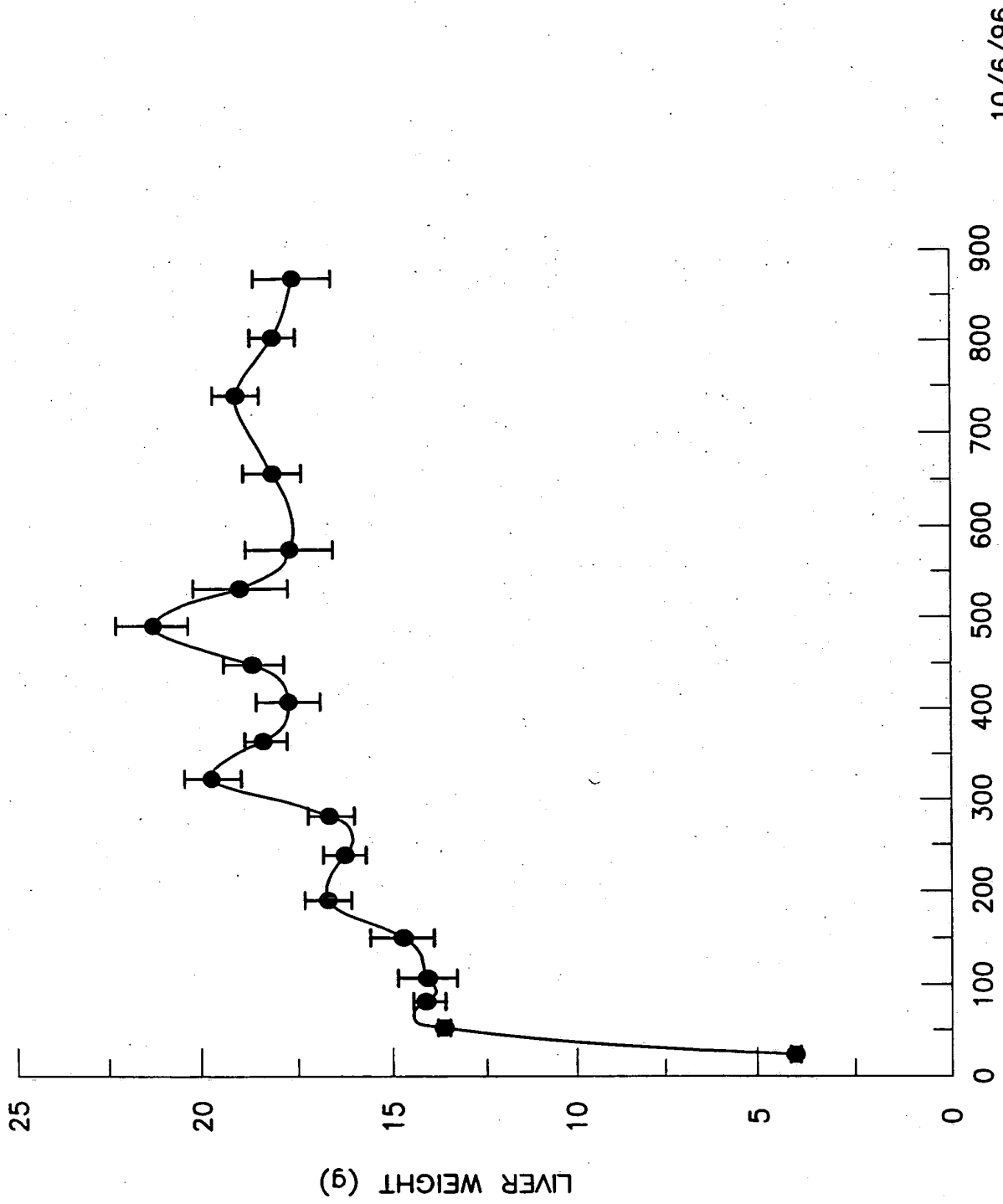


FIG. 2: LIVER WEIGHTS AS A FUNCTION OF AGE

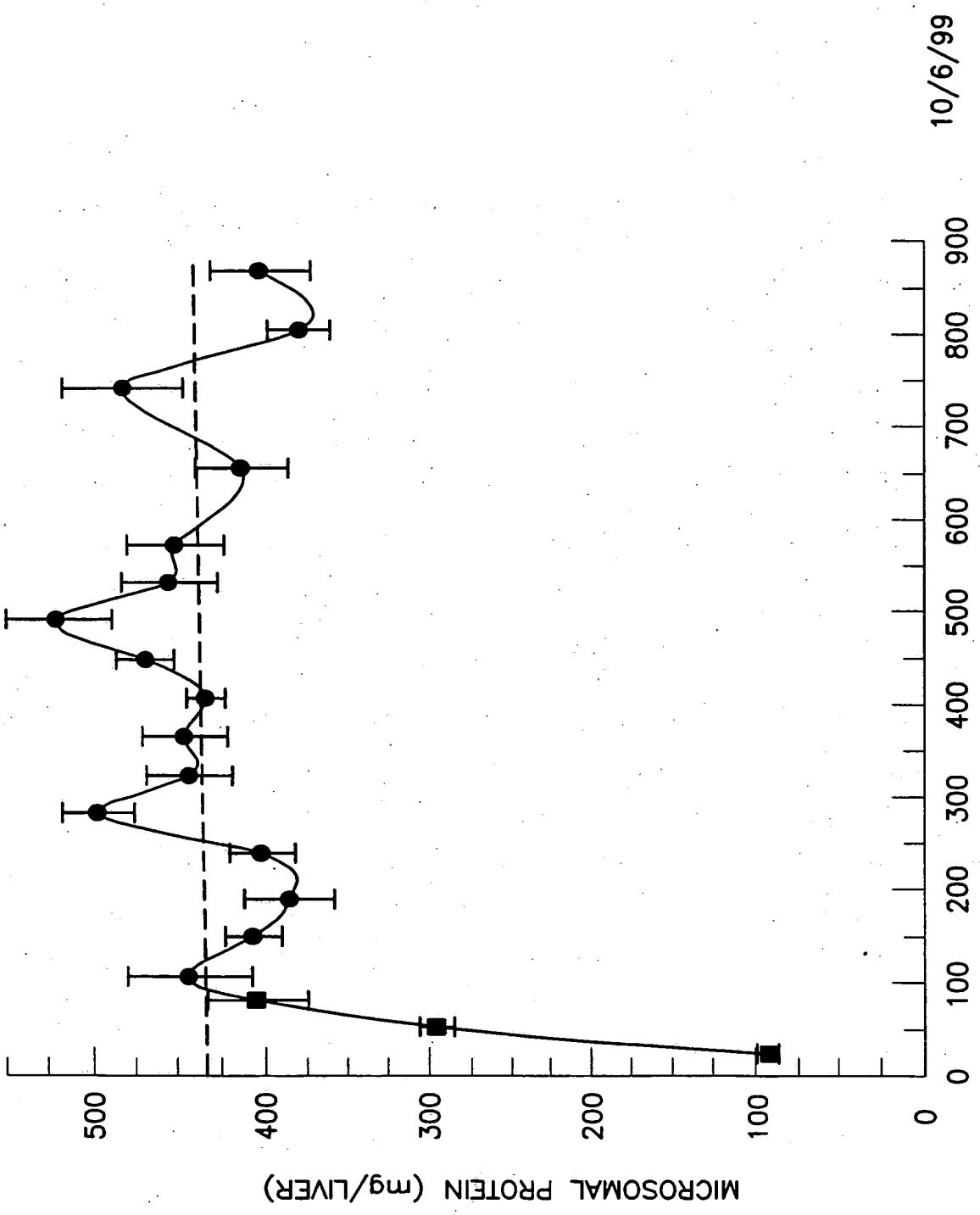


FIG. 3: MICROSOMAL PROTEIN PER GRAM LIVER AS A FUNCTION OF AGE

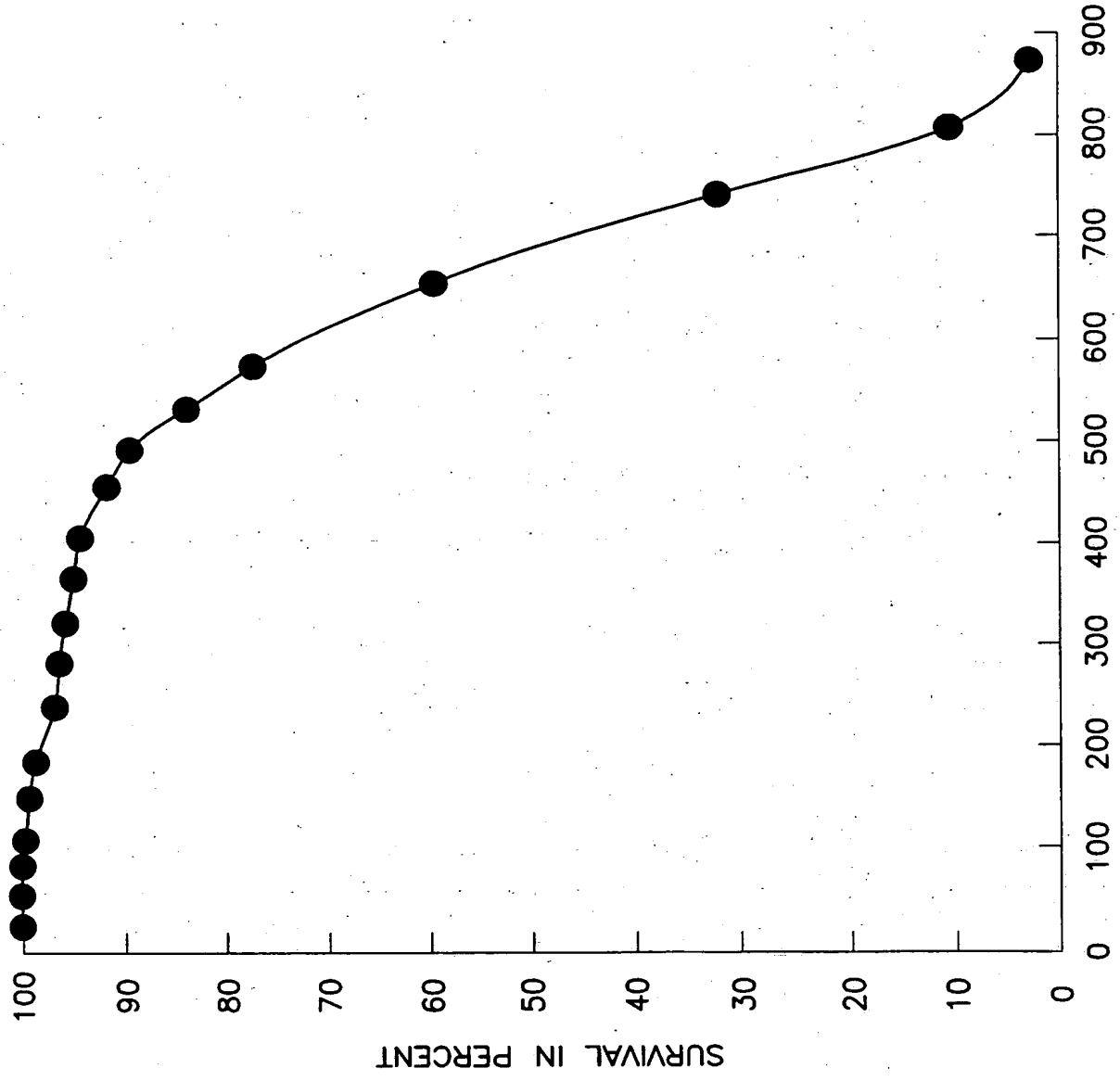


FIG. 4: LIFE TABLE ANALYSIS OF ANIMAL SURVIVAL

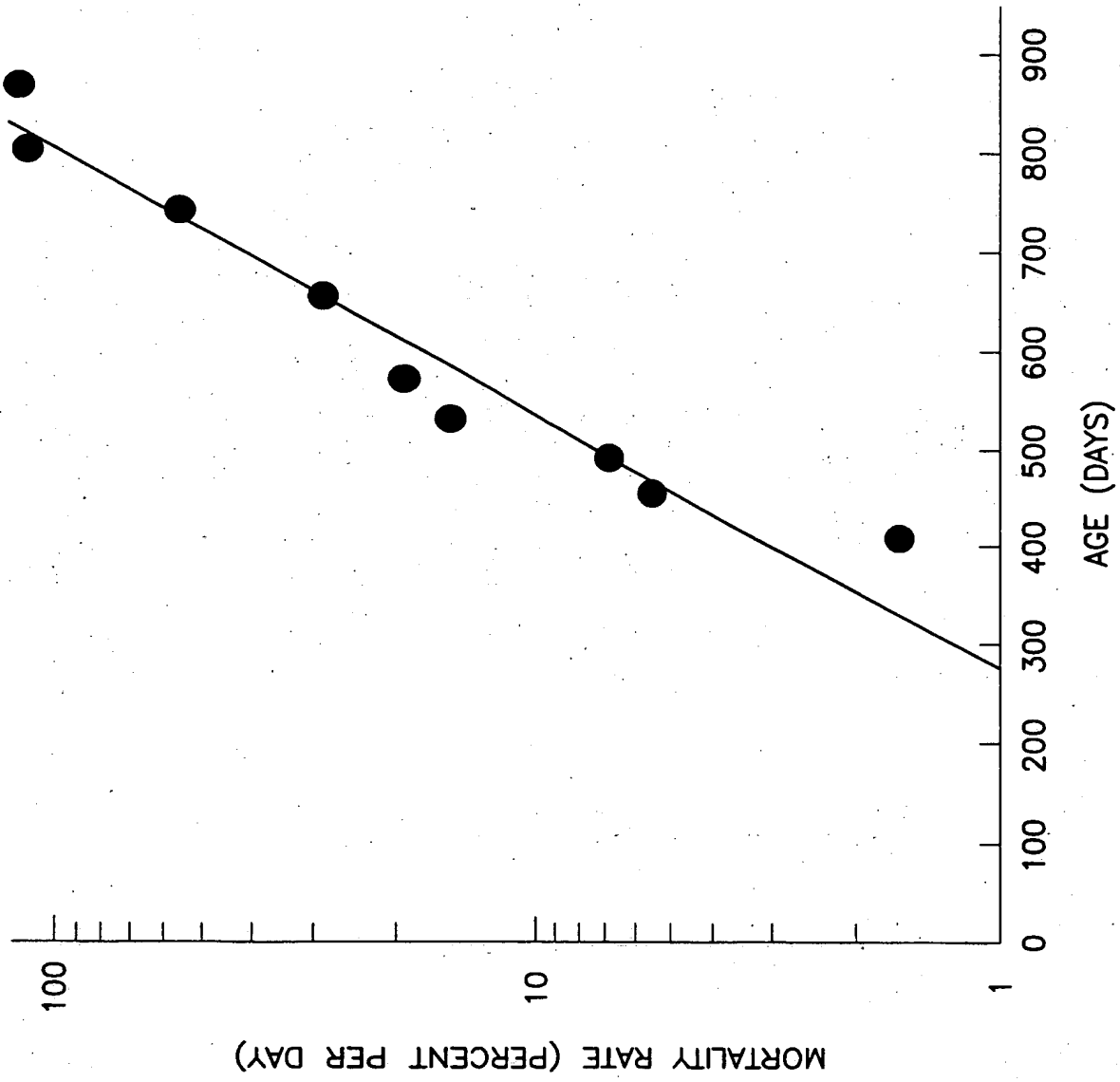


FIG. 5: MORTALITY RATE VERSUS AGE

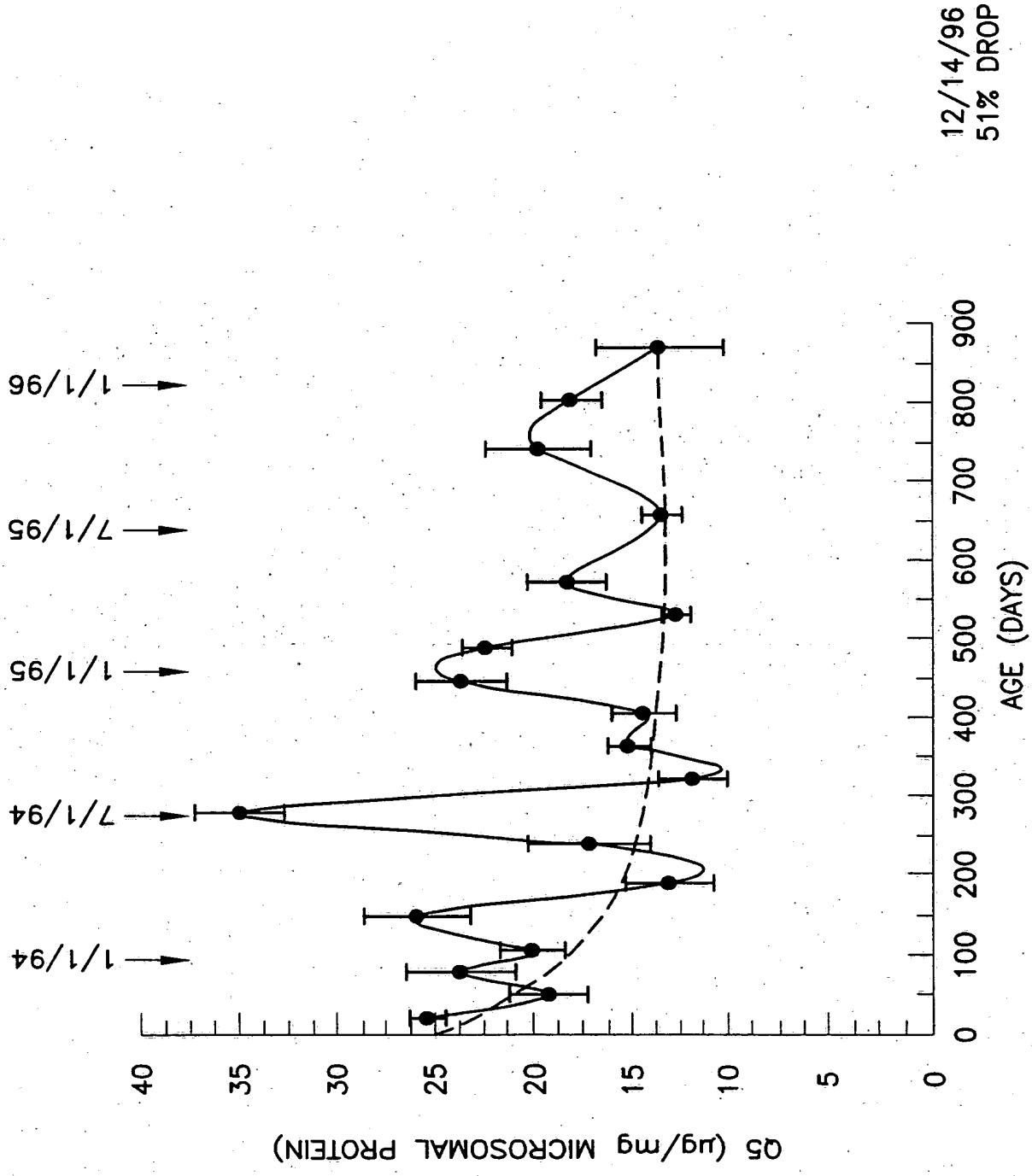


FIG. 6: EFFECT OF AGE ON THE HEPATIC, MICROSOMAL Q5 LEVELS

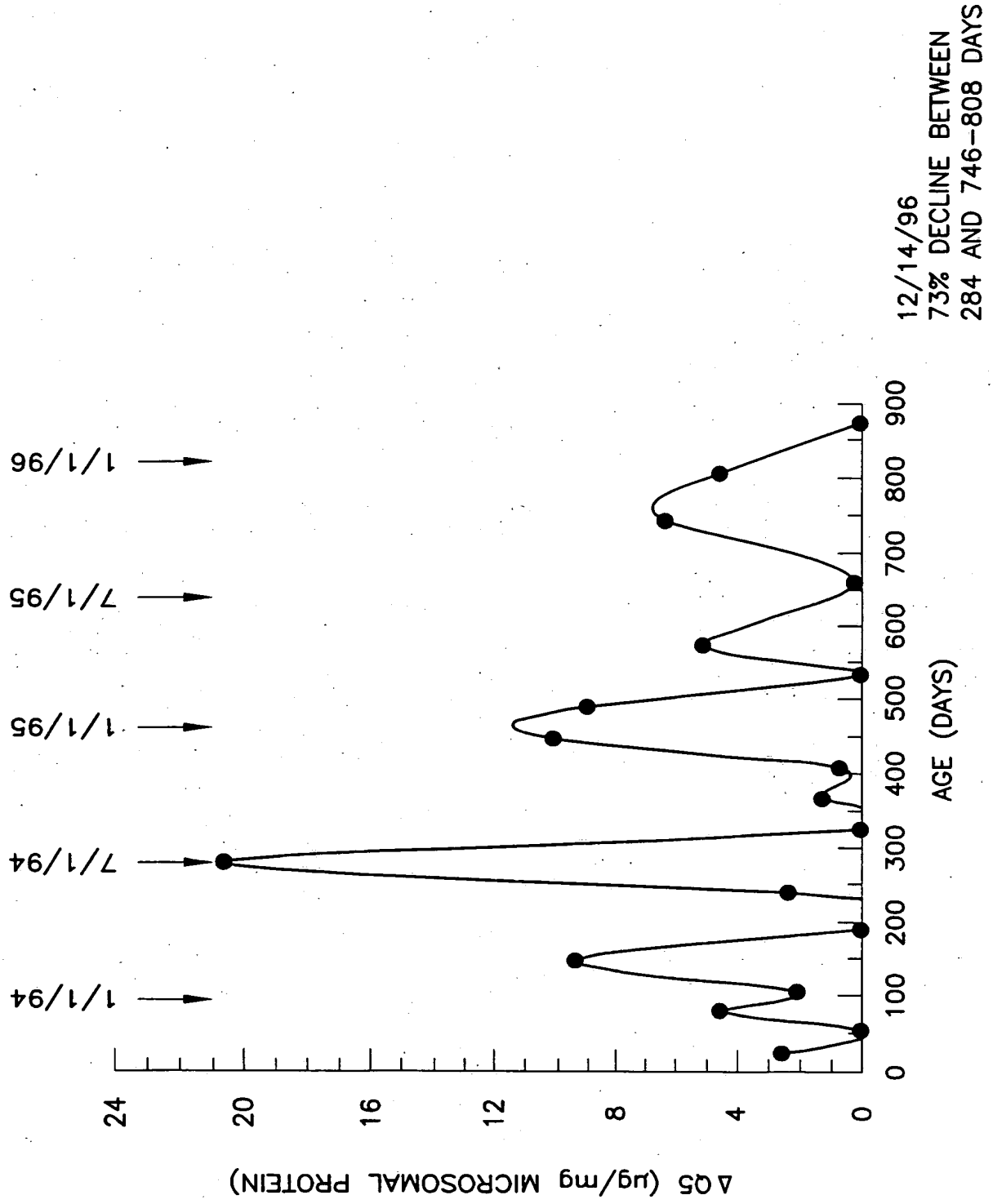


FIG. 7: THE EFFECT OF AGE ON THE STRESS RESPONSIVE LEVELS OF Q5

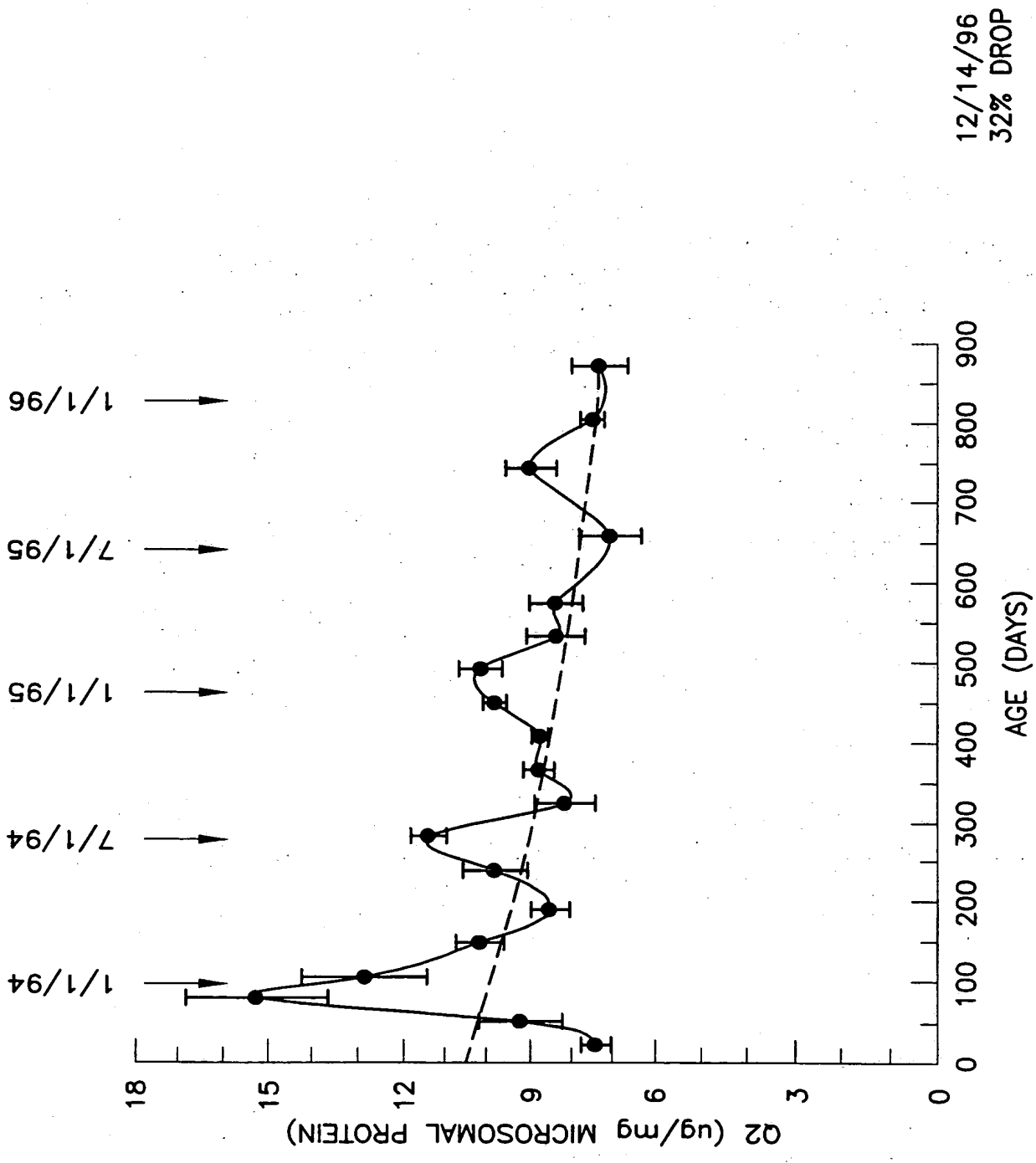


FIG. 8: EFFECT OF AGE ON THE HEPATIC, MICROSOMAL Q2 LEVELS

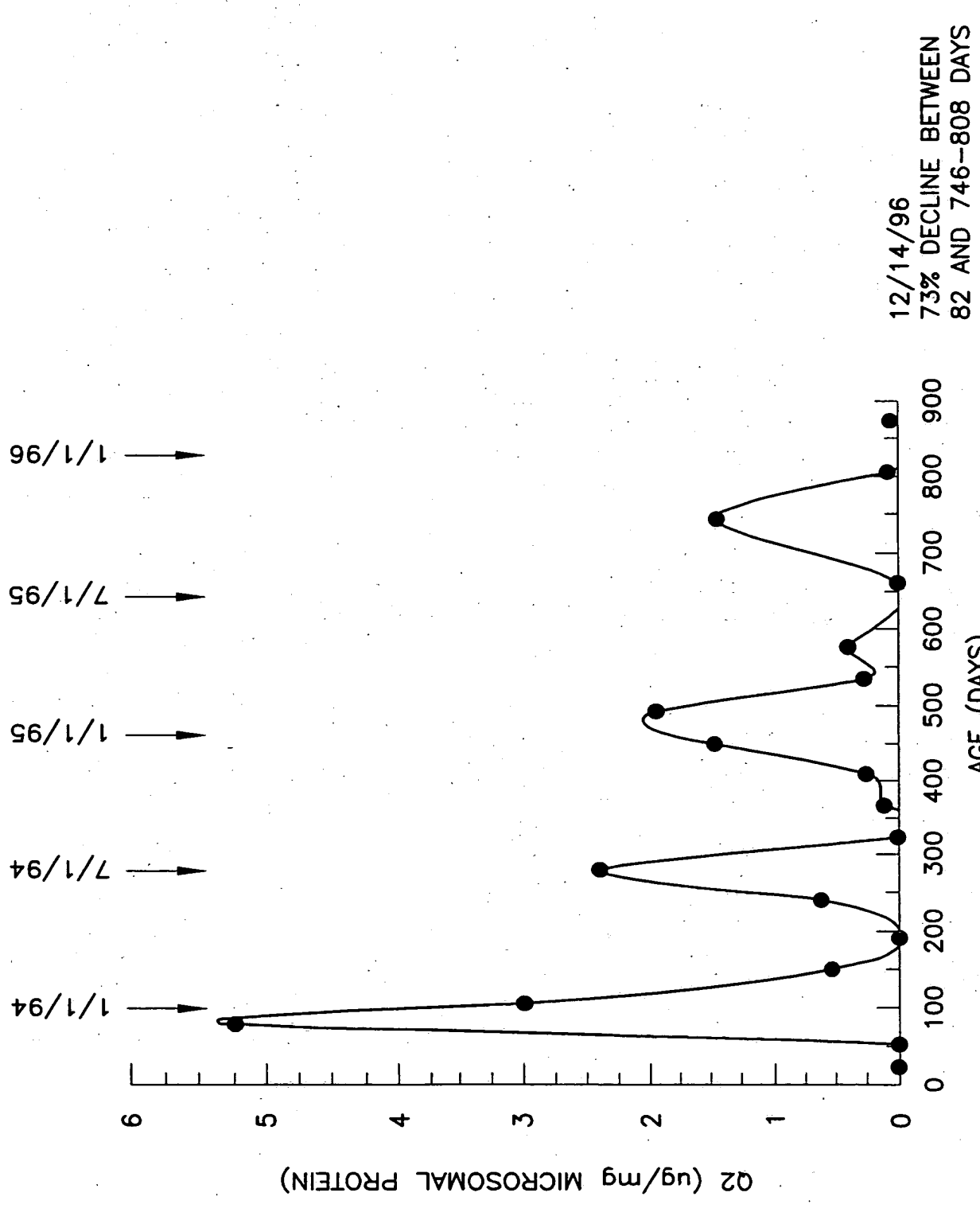


FIG. 9: THE EFFECT OF AGE ON THE STRESS RESPONSIVE LEVELS OF Q2.

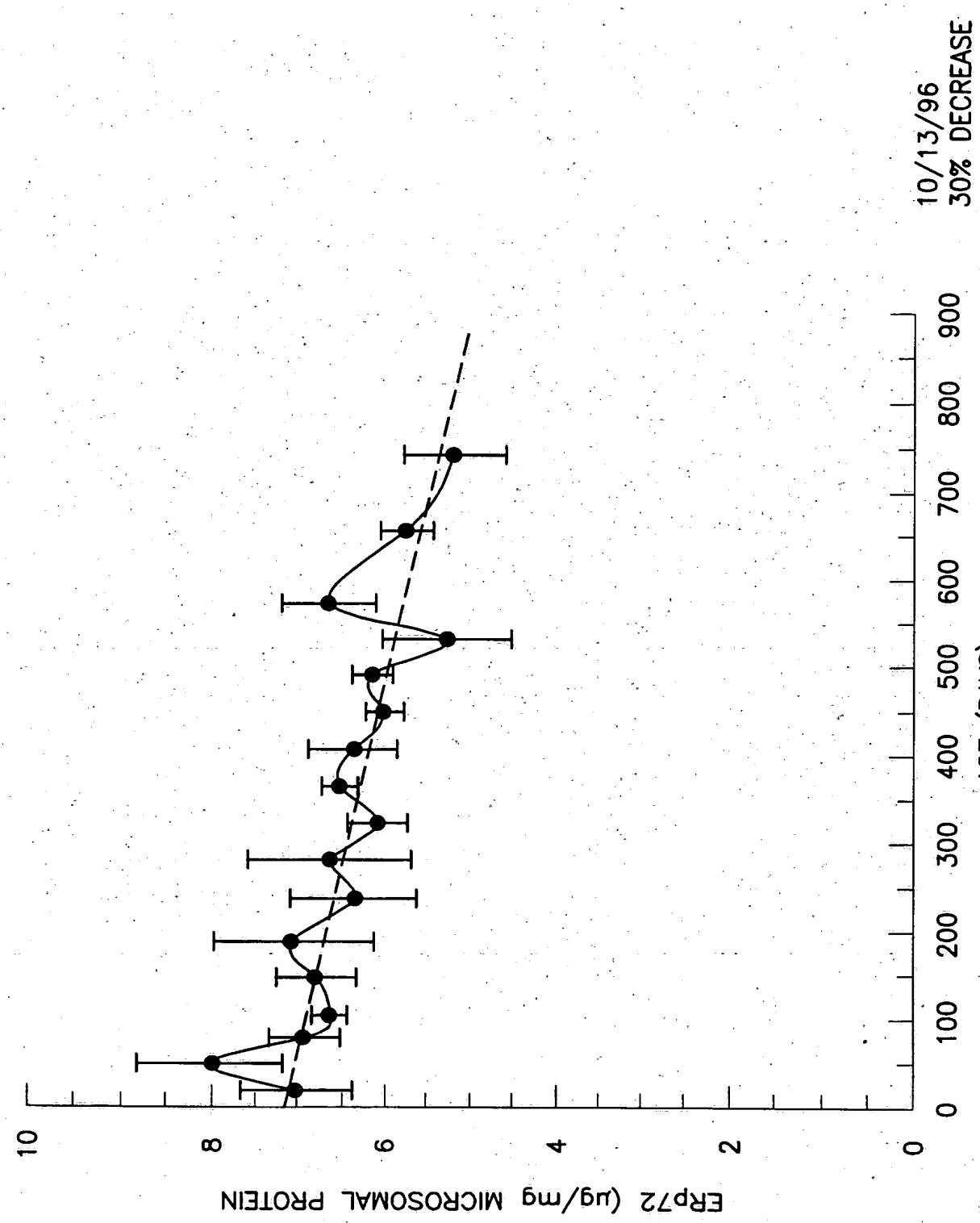


FIG. 10: EFFECT OF AGE ON THE HEPATIC, MICROSOMAL ERp72 LEVELS

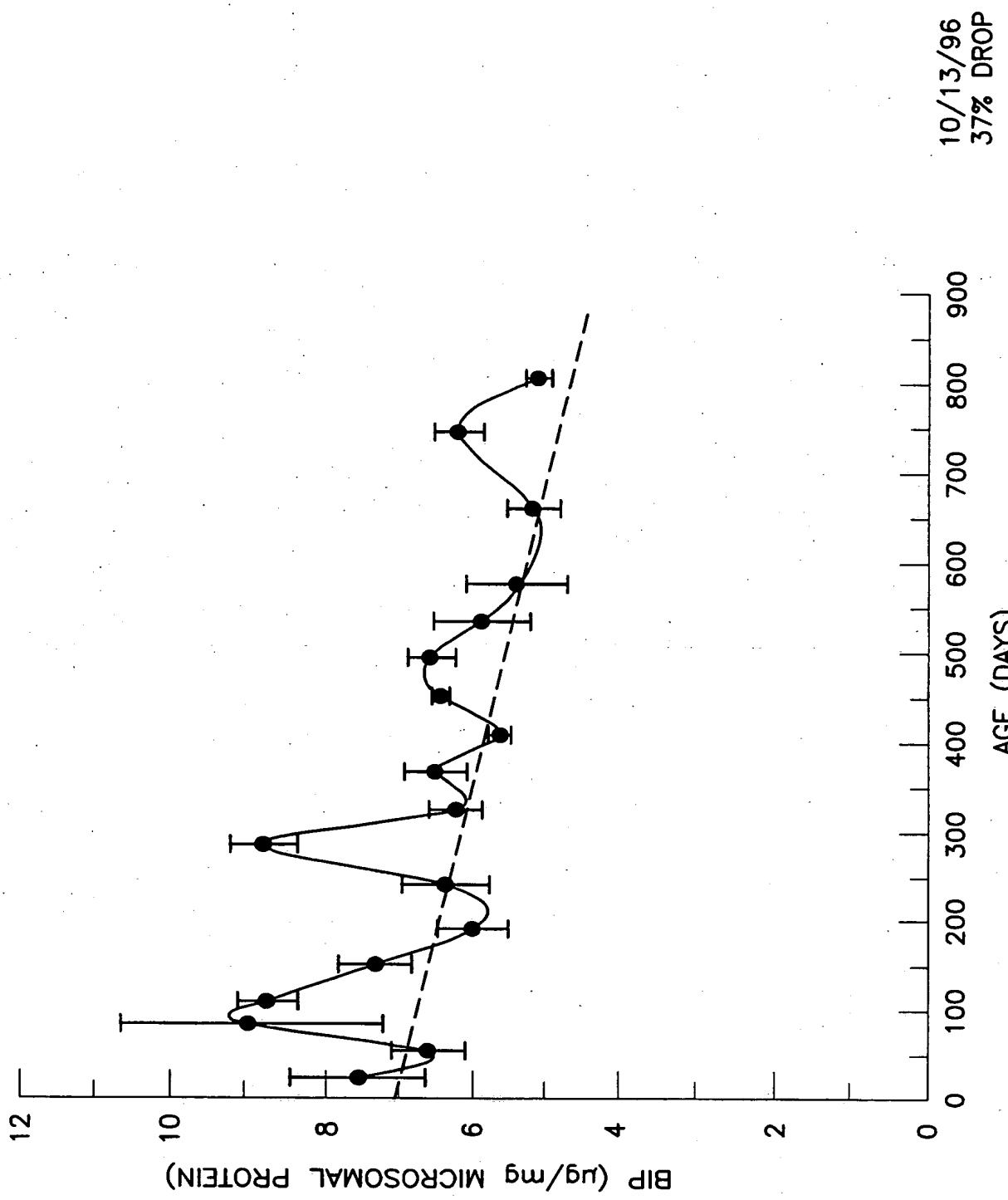


FIG. 11: EFFECT OF AGE ON THE HEPATIC, MICROSOMAL BIP LEVELS

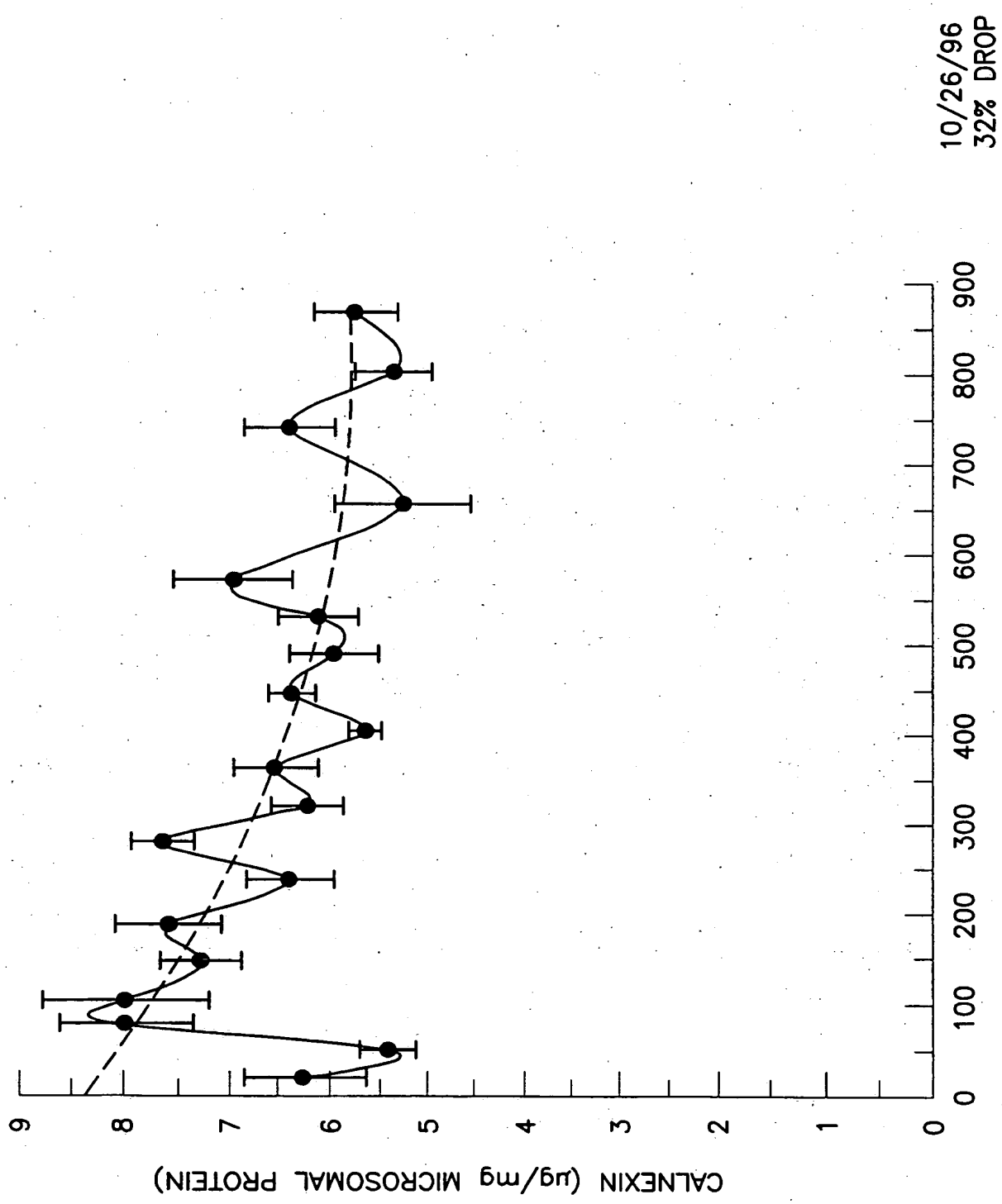


FIG. 12: EFFECT OF AGE ON THE HEPATIC, MICROSOMAL CALNEXIN LEVELS

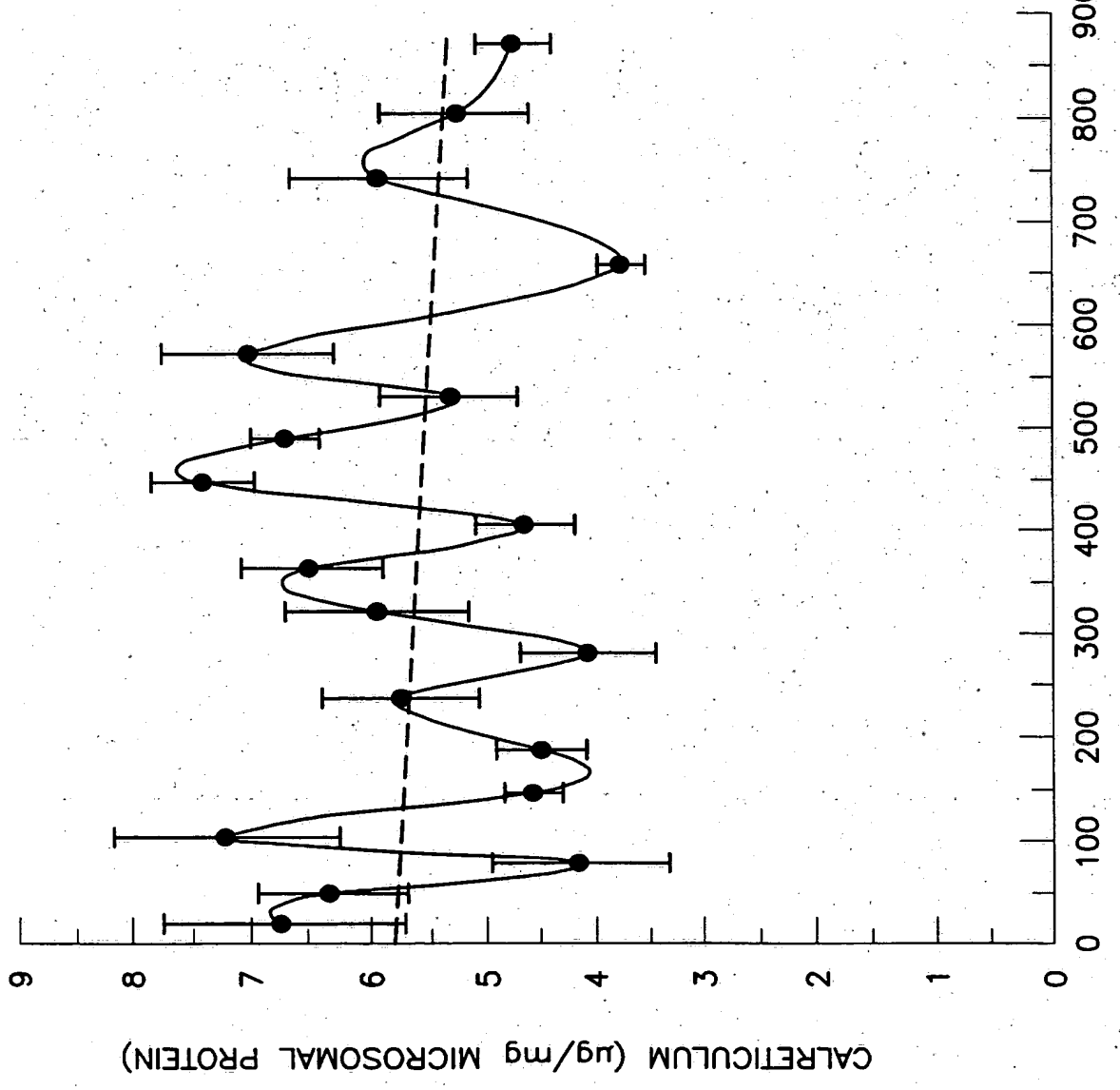


FIG. 13: EFFECT OF AGE ON THE HEPATIC, MICROSOMAL CALRETICULIN LEVELS

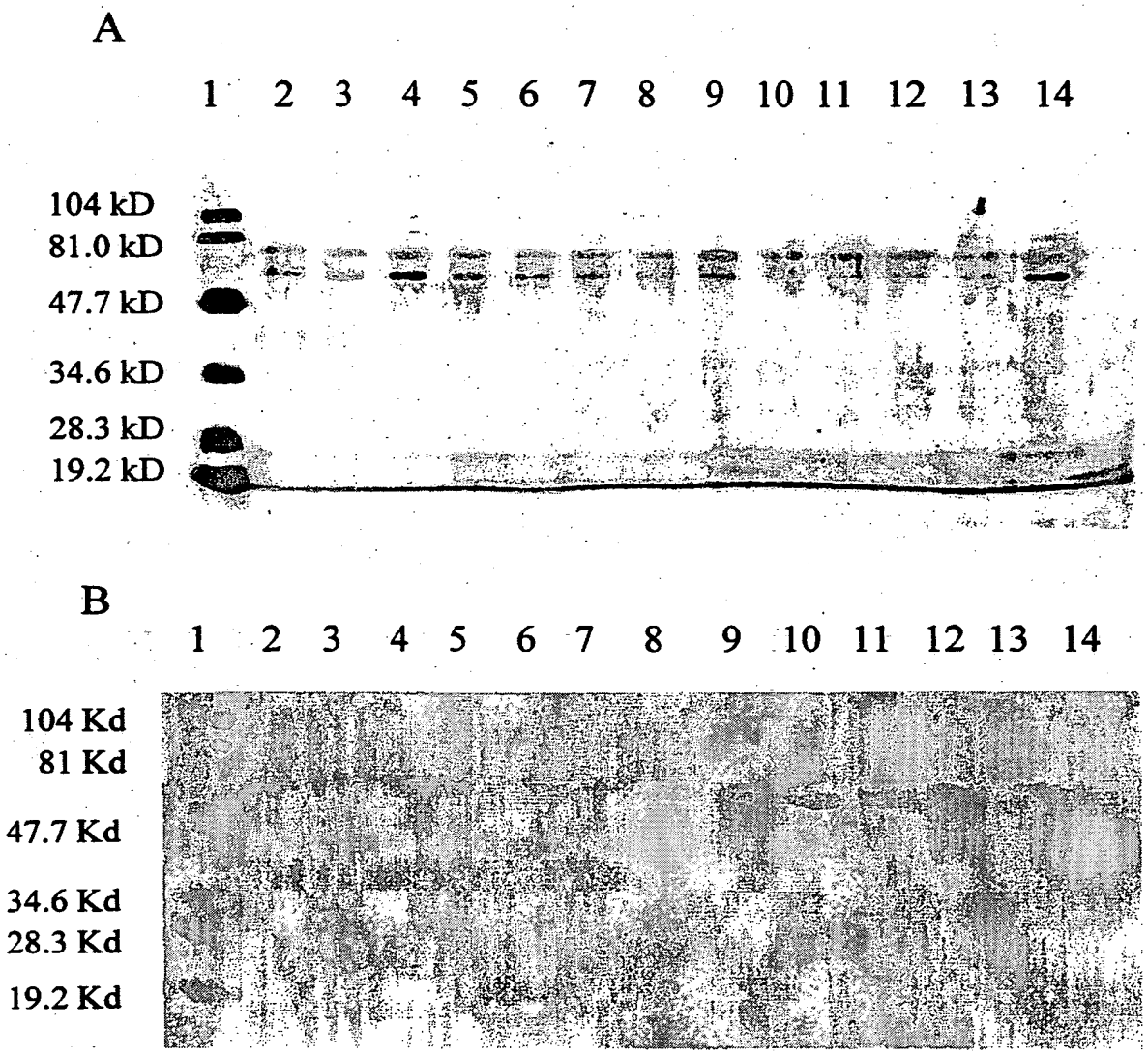


FIGURE 14

Immunoblots with A) Chicken Polyclonal antibodies to Q2 and B) Monoclonal Antibodies to B-amyloid 1-42. Channel 1-Prestrained Standards; Channels 2-Rat, Hepatic Microsomes; Channels 3-7 & 9-14--15µl of CSF from Normal,Human Subjects,Ages 6 Months to 59 Years; Channel 8 - Purified Q2

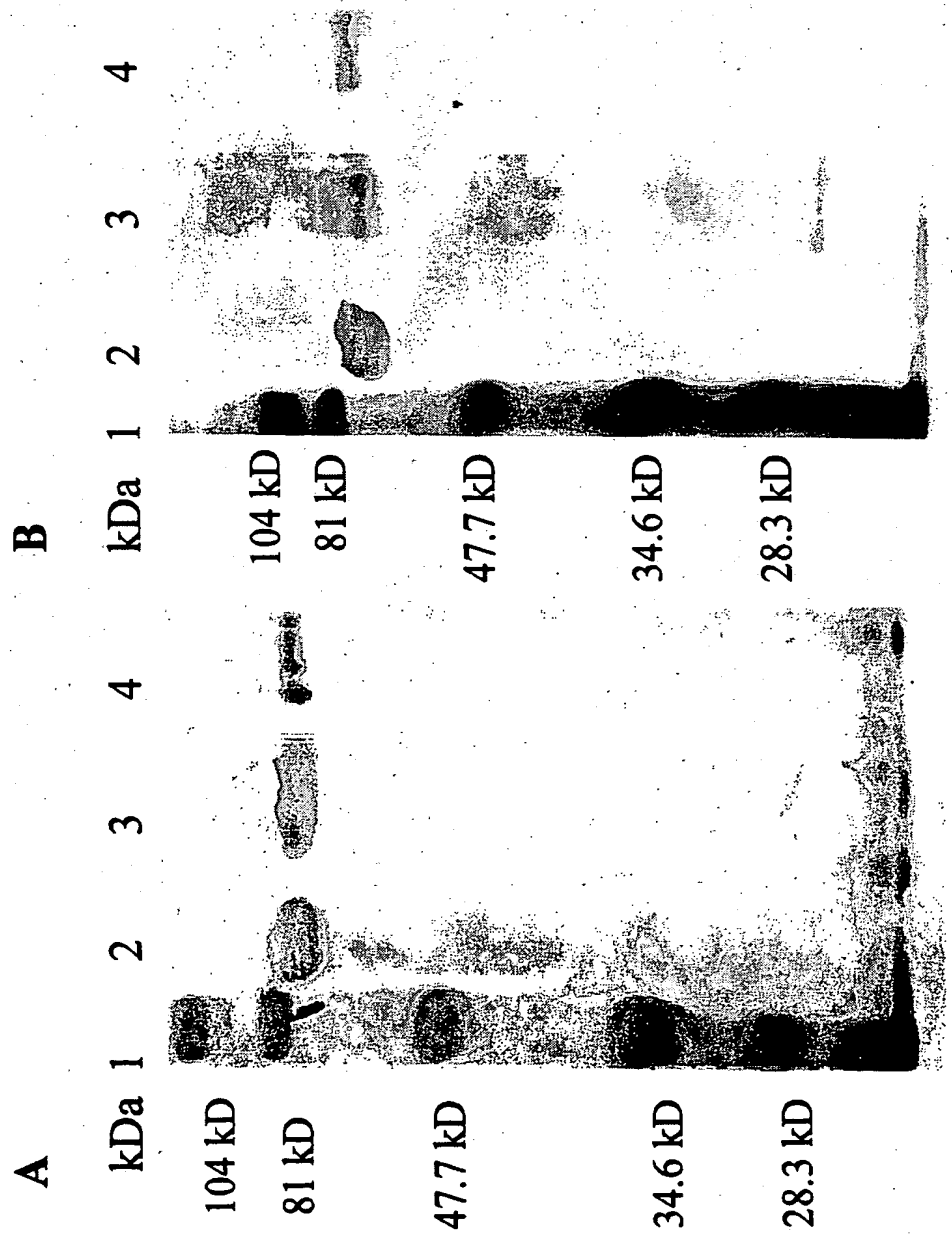


FIGURE 15

Immunoblots with A) Polyclonal antibodies to q2 and B) to B-amyloid 1-42. Channel 1 Prestained Srandards; Channels 2-CSF; Channel 3-Seperated by afinity chromatography with anti-B-amyloid antibodies; Channel 4-CSF Seperated by affinity chromatography with anti-Q2 antibodies

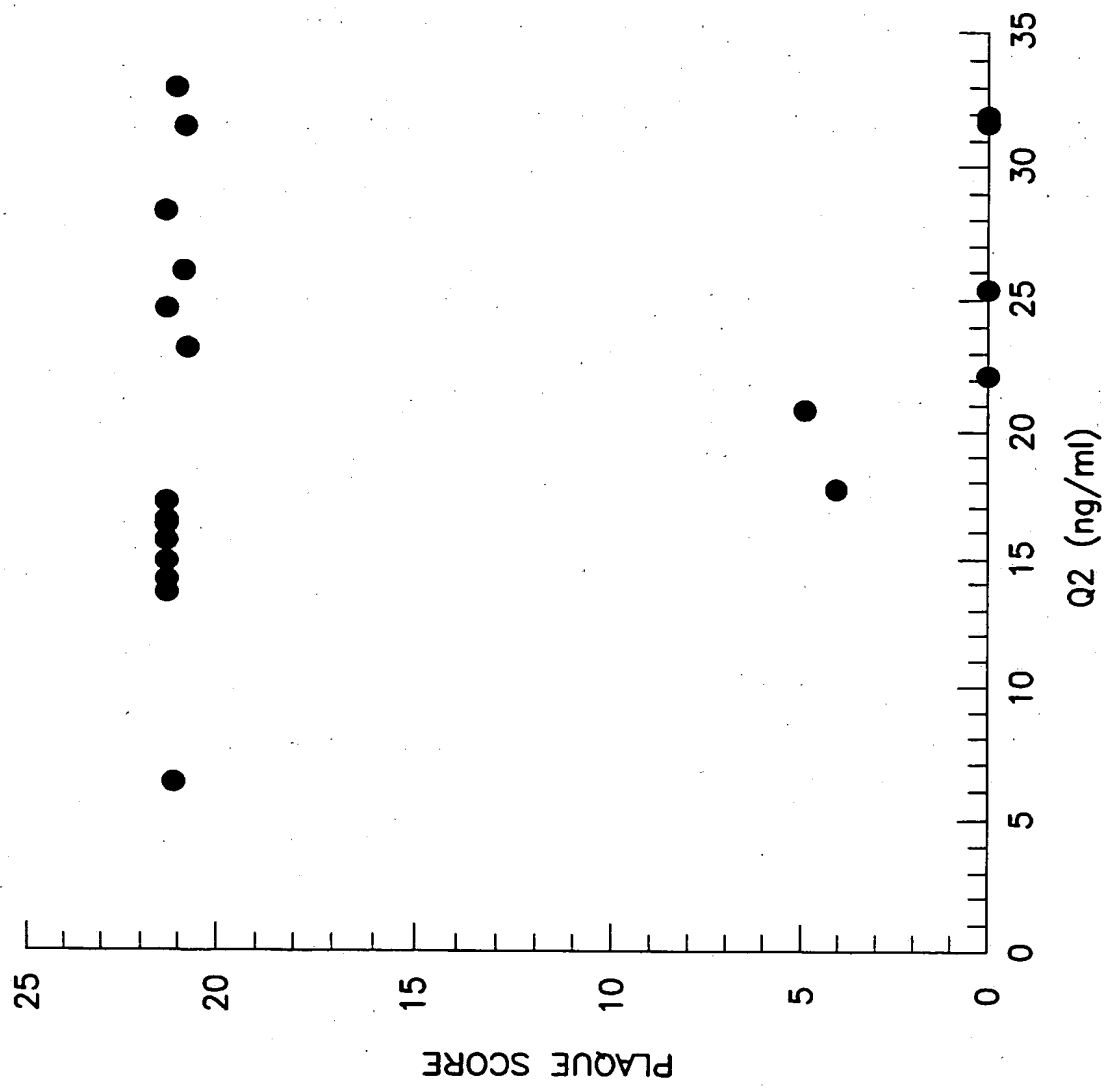


FIG. 16: THE CSF CONCENTRATIONS OF Q2 COMPARED TO SENILE PLAQUE SCORES IN THE BRAINS OF PARTICIPANTS IN THE NUN STUDY.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25593

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US November 1998 (1998-11) HOLTZMAN J L ET AL: "A chaperon binds to b-amyloids: Implications for the etiology of Alzheimer's disease." Database accession no. PREV199900015705 XP002139087 abstract & 38TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CELL BIOLOGY;SAN FRANCISCO, CALIFORNIA, USA; DECEMBER 12-16, 1998, vol. 9, no. SUPPL., November 1998 (1998-11), page 106A Molecular Biology of the Cell Nov., 1998 ISSN: 1059-1524 --- -/--	1-3,12, 22,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	19. 06. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Goetz, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/25593

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 780 587 A (POTTER HUNTINGTON) 14 July 1998 (1998-07-14) column 3, line 44 -column 10, line 6; claims 1-5 column 17, line 1-42 column 18, line 47 -column 19, line 44 column 21, line 34-67 column 22, line 31-34 claims 7,8; examples 13,14	4-7,36, 37
X	EP 0 783 104 A (ORIENTAL YEAST CO LTD) 9 July 1997 (1997-07-09) claim 8; example 2	36,37
X	IDA ET AL.: "Analysis of heterogeneous βA4 Peptides in Human Cerebrospinal Fluid and Blood by a Newly Developed Sensitive Western Blot Assay" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996 (1996-09-13), pages 22908-22914, XP002138701 page 22909, right-hand column, last paragraph -page 22910, left-hand column, paragraph 2	36,37
A	WO 96 12736 A (POTTER HUNTINGTON ;HARVARD COLLEGE (US)) 2 May 1996 (1996-05-02) page 3, line 31 -page 4, line 12 page 10, line 11-16	1-37
A	MATSUBARA ET AL.: "Apolipoprotein J and Alzheimer's amyloid β solubility" BIOCHEMICAL JOURNAL, vol. 316, 1996, pages 671-679, XP002138702 page 671, right-hand column, paragraph 1 page 678, right-hand column, last paragraph	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/25593

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 33 - 35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/25593

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780587 A	14-07-1998	US 5506097 A	09-04-1996
		US 5338663 A	16-08-1994
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997
		WO 9612736 A	02-05-1996
		EP 0623146 A	09-11-1994
		JP 7509447 T	19-10-1995
		WO 9315112 A	05-08-1993
		EP 0546101 A	16-06-1993
		JP 6500560 T	20-01-1994
		WO 9203474 A	05-03-1992
EP 0783104 A	09-07-1997	JP 9178743 A	11-07-1997
WO 9612736 A	02-05-1996	US 5780587 A	14-07-1998
		CA 2203825 A	02-05-1996
		EP 0787146 A	06-08-1997